Acta
SOCIETATIS MEDICORUM UPSALIENSIS

VOL. LXIX. SUPPLEMENTUM 7

1964

L Braman

ALMQVIST & WIKSELL

UPPSALA/STOCKHOLM

ACTA SOCIETATIS MEDICORUM UPSALIENSIS

NEW SERIES. VOL. 69

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Uppsala, Sweden.

CHROMATOGRAPHIC AND MAGNETIC STUDIES ON HUMAN CERULOPLASMIN

BY

LARS BROMAN

STOCKHOLM 1964

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PREFACE

This investigation was initiated largely through the encouragement received from my teacher in clinical psychiatry, Prof. B. Jacobowsky, Uppsala. The work was carried out at the Institute of Biochemistry, University of Uppsala, and I am much indebted to Prof. A. Tiselius for his stimulating interest and helpful advice throughout the progress of this investigation, and for placing the excellent resources of this Institute at my disposal. My thanks are due to my teacher, Prof. Bo G. Malmström, now Head of the Department of Biochemistry, University of Gothenburg, with whom I had the privilege to collaborate in several of the studies to be described in the following. I am also much indebted to Dr. Hans G. Boman, at the Institute of Biochemistry, Uppsala, for numerous stimulating discussions and helpful suggestions.

I wish to thank my collaborators, Drs. Kerstin Stråby (*née* Kjellin), Institute of Biochemistry, Uppsala, T. Vänngård and R. Aasa, Institute of Physics, Uppsala, A. Ehrenberg, Department of Biochemistry, Karolinska Institutet, Stockholm, and A. G. Bearn, the Rockefeller Institute, New York.

I wish to express my sincere gratitude to Prof. T. S:son Frey, the Psychiatric Clinic, Uppsala, to Prof. H. Forssman, Ulleråker Hospital, to Prof. G. Ågren and to Dr. T. Laurent, at the Institute of Medical Chemistry, for their kind interest in this work.

I would also gratefully acknowledge the skilful help by Miss K. Ohlson, Mrs. I. Ekstrand and Mr. S.-O. Falkbring who assisted in the laboratory, by Mr. G. Forsling who made the drawings, by Drs. R. Elston and J. Gordon who revised the English text, and by Mrs. B. Björklund who typed the manuscript.

The work was supported financially by Mrs. S. Thånell, Malmö, by grants from the Swedish Medical Research Board, from the Medical Faculty, Uppsala, from Thånellska Minnesfonden för Psykiatrisk forskning, from the Division of General Medical Sciences, U.S. Public Health Service, R.G.-6542-3 (through Prof. B. G. Malmström), from EIR Insurance Company, Stockholm, and from AB Kabi, Stockholm. I am, in addition, much obliged to Mr. H. Björling and AB Kabi for generous gifts of ceruloplasmin.

Thanks are due to copyright owners who have kindly agreed to my use of figures and tables from earlier writings.

Uppsala, April, 1964.

Lars Broman.

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INTRODUCTION

Plasma of the healthy adult human individual contains, on the average, 114 μg of copper per 100 ml, the normal range being 68—165 μg (1). About 95 per cent of this copper belongs to a characteristic blue metalloprotein, ceruloplasmin. This protein was isolated and characterized by Holmberg and Laurell (2—8), and their thorough analysis has been supplemented by a large body of physico-chemical and physiological data (reviews in refs. 8—11). Thus, to mention a few, ceruloplasmin is probably synthesized in the liver (12), it contains 8 copper atoms per molecule (4) and about 7.8 per cent carbohydrates (13). The molecular weight is 160,000 (14). The serum mean concentration is 35 mg/100 ml in the normal adult and 10 mg/100 ml in the normal newborn (15). Increased plasma concentrations are observed in a variety of conditions, including normal pregnancy, certain acute and chronic inflammatory diseases, neoplasma, and various forms of anemia (9).

But the function of ceruloplasmin remains virtually unknown, in spite of the wealth of facts. There are, in addition, two circumstances that are thought to be particularly important for the understanding of the protein's role. Firstly, subnormal plasma concentrations, though generally quite uncommon after the first few months of life, are a frequent occurrence in hepatolenticular degeneration (Wilson's disease). Secondly, ceruloplasmin catalyses a number of oxidative reactions with molecular oxygen as the electron acceptor.

Hepatolenticular degeneration is a rare disorder which is inherited in a recessive autosomal fashion. Since 1912, when the entity was first recognized (16), about 600 cases have been recorded (reviews in refs. 9, 11, 17 and 115). The malady appears to be the only instance of primarily deranged copper metabolism in man. Its patho-physiological mechanism is obscure. The condition is characterized biochemically by an increased uptake of alimentary copper, by abnormal deposits of the metal in various organs, notably the liver, the brain, and the kidneys, and by increased excretion of the element in the urine. The non-ceruloplasmin copper fraction in plasma is increased but the ceruloplasmin deficit is generally sufficiently large to make the total plasma copper concentration subnormal. Certain observations indicate that the synthesis of ceruloplasmin may be impaired in Wilson's disease (12). However, a small number of otherwise typical cases are known in whom the ceruloplasmin concentration was within or close to the normal range (18). Thus, if certain signs and symptoms of the disease are caused by a failing ceruloplasmin function, then the protein may be qualitatively abnormal in this small group (1, 19).

The catalytic property of ceruloplasmin was demonstrated by Holmberg and Laurell (6) who found that *p*-phenylene diamine, hydroquinone, catechol, pyrogallol, dioxyphenylalanine, adrenaline, and ascorbic acid are substrates. The list of electron donors that can partake in ceruloplasmin-catalyzed oxidations has subsequently been augmented with the *N,N*-dimethyl (20) and the *N*-substituted tetramethyl (21) derivatives of *p*-phenylenediamine, and, furthermore, with noradrenaline (22), serotonin (23) and others (see, for example, ref. 24). The activity of ceruloplasmin is, however, generally low compared with a typical oxidase and the best substrates (24) are non-physiological.

The scope of the present work is 1) to analyze human ceruloplasmin, using chromatographic techniques, for signs of qualitative variation in the absence and in the presence of Wilson's disease, and 2) to investigate the role of copper in the catalytic action of the protein.

Several of the results described in the following have been presented elsewhere. The reader is referred to refs. 25—30 for additional details.

ABBREVIATIONS

AMP	adenosine monophosphate
ATP	adenosine triphosphate
D.C.	direct current
DEAE	Diethyl aminoethyl-
DPP	*N,N*-dimethyl-*p*-phenylene diamine
EDTA	ethylene diamine tetraacetic acid
ESR	electron spin resonance
NADH	reduced form of nicotinamide adenine dinucleotide
PPD	*p*-phenylene diamine
TEA	triethylamine
TPP	*N,N,N′,N′*-tetramethyl-*p*-phenylene diamine
Tris	tris(hydroxymethyl)aminomethane

CHAPTER 1

LOCATION OF CERULOPLASMIN IN HYDROXYLAPATITE CHROMATOGRAMS

Introduction

In 1957 Uriel *et al.* (31, 32, 33) demonstrated a heterogeneity of purified human ceruloplasmin when analyzed by agar gel electrophoresis. Two copper-containing components with α_1 and α_2 mobility appeared, both being catalytically active with *p*-phenylene diamine as substrate (Fig. 1). The two components were identical according to immunoelectrophoresis, and the precipitation lines contained copper and displayed catalytic ac-

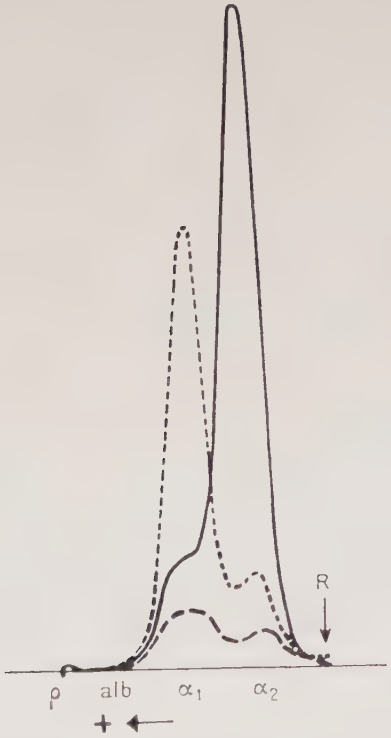

Fig. 1. Agar gel electrophoresis of purified ceruloplasmin from human serum. Densitometric curves obtained of 1) coloured oxidation product of *p*-phenylene diamine (full line), 2) protein staining with Amidoschwarz (pointed line), and 3) copper staining with Alizarin Blue S (dashed line). R = starting point (32).

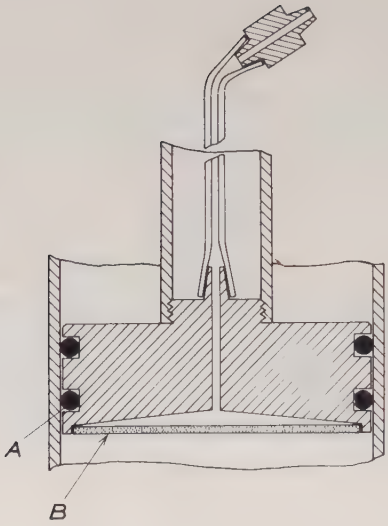

Fig. 2. Section of top piston of plexi-glass column tube used in most gradient chromatographies. A, 0-ring, B porous polystyrene disc (30).

tivity. The content of copper and protein was higher in the α_1 component, whereas the α_2 component was catalytically more active (Fig. 1). Individual sera (including three Wilson sera) that were analyzed by the same methods showed only one active component.

The minor active component discovered by Uriel and collaborators may either be an artifact produced in the purification procedure or a natural ceruloplasmin variant whose concentration in serum samples is too low to be detectable with the methods employed. In order to approach this problem and to make possible further analyses of subfractions, a separation method is required that is superior to agar gel electrophoresis, both as regards load capacity and resolving power. Such a method is gradient chromatography on hydroxylapatite. With this system the author obtained two ceruloplasmins from fresh human serum (25, 26) and three ceruloplasmins from purified preparations. A colourimetric method, based on the catalytic activity of ceruloplasmin, was devised in order to facilitate simultaneous analyses of a comparatively large number of chromatographic fractions.

Material and methods

a) *Anlaytical methods*

The protein content was measured as the absorbancy at 280 mμ in a Hilger Uvispec spectrophotometer. 0.5 and 0.1 cm cells were used, and,

when necessary, dilutions were carried out to keep the absorbancy below 1.2. When possible, ceruloplasmin concentrations were determined as the absorbancy at 610 mμ in a 1 cm or 2 cm cell.

The catalytic activity of ceruloplasmin was determined with *N,N*-dimethyl-*p*-phenylene diamine (DPP) as substrate (20). Both the monohydrochloride (Eastman) and the dihydrochloride (No. 3067, Merck, Darmstadt) were tested. The latter was obtainable in a less auto-oxidized state, and was used in the standard procedure of the method. Stock solution I was prepared by dissolving 3.120 g of DPP dihydrochloride in 50 ml of 4 *N* hydrochloric acid and diluting with distilled water to 100 ml. Solution I was stored in plastic tubes (slightly more than 1 ml in each) at about —20°C, and was usable for more than one year. Stock solution II was triethylamine-acetic acid buffer, pH 5.5 at 20°C (0.50 *M* with respect to triethylamine, 0.58 *M* with respect to acetic acid). Stock solution III was a 0.01 *M* solution of the disodium salt of ethylene diamine tetraacetic acid (Hopkin and Williams Ltd., Chadwell Heath, Essex, England). Immediately before use, the substrate solution was made up from the following: 1 ml of each of stock solutions I and III, 50 ml of stock solution II (+4°C) and distilled water (+4°C) to a volume of 250 ml. The final pH was about 5.1 at 37°C. Depending on the range of the oxidase concentration expected in starting material or chromatographic fractions, aliquots of 10 to 200 μl were pipetted into suitable test tubes. Using a semiautomatic syringe (34), 2 ml of the cold substrate solution was rapidly distributed into each test tube. The whole set of tubes was then placed in a 37°C thermostat for 10 min. and afterwards transferred to ice water, where it remained during the reading of the extinction at 552 mμ. A 1.0 cm semimicrocell and a Beckman *B* spectrophotometer was used. Up to 84 samples were measured simultaneously, including 8—12 evenly distributed blanks and, occasionally, 6 standard samples of ceruloplasmin (AB Kabi, Stockholm).

b) *Starting material*

Sera for individual analysis were obtained from normal donors or from somatically healthy, fasting in-patients at a psychiatric clinic. About 50 ml of blood was tapped from the cubital vein by means of an evacuated nonsterile flask connected to the needle *via* a polyvinyl tubing. The sample was kept at room temperature for about one hour and in a refrigerator for about one day, after which serum was collected and dialyzed in a cold room (about +4°C) against starting buffer for about 20 hours.

Pooled retroplacental serum, not older than two or three days and stored unfrozen in a cold place, was filtered through a 1—2 cm layer of Pevicon on a Büchner funnel. Pevicon is a copolymer of polyvinyl chloride and polyvinyl acetate (C-870, Fosfatbolaget, Stockholm 5). The powder was pre-treated with glacial acetic acid followed by frequent washings

in distilled water to eliminate traces of ultraviolet-absorbing material. The starting concentration of phosphate was achieved by dilution of the 1.0 *M* stock solution with serum.

Several preparations of purified ceruloplasmin manufactured by AB Kabi, Stockholm (35) and one produced by Squibb and Sons, New York, were prepared for chromatography by dialysis against the buffer of the starting concentration. (I am indebted to Dr. J. H. Pert of the American Red Cross, Washington, D.C., for the Squibb preparation).

c) *Chromatographic methods*

Buffers were prepared by dilution from a stock solution of 1.0 *M* potassium phosphate. The latter was made up from equal parts of primary and secondary phosphate, which means that in the actual elution range used in these experiments, 0.125 *M* to 0.50 *M*, the pH was about 6.8. Hydroxylapatite was prepared according to Tiselius *et al.* (36). Cf. also refs. 37 and 38. The apatite was equilibrated with buffer of the starting concentration, and finely dispersed crystals were carefully removed before the column tube was filled. In a few experiments (*e.g.* Fig. 9) the apatite was mixed with about equal parts of Pevicon in an attempt to improve the flow rate. The same apatite column could be used repeatedly, if protected from running dry. The final buffer concentration (0.5 *M*) was sufficient to desorb all serum proteins. Renewed equilibration with buffer of the starting concentration (0.125 *M*) could thus be undertaken directly after a run. The passage of about ten times the void volume of this buffer was sufficient to obtain equilibrium. No serious shrinkage of the adsorbent or obstruction of the flow due to swelling was observed with the indicated range of buffer concentrations.

Plexiglass column tubes, of a construction (cf. ref. 39) calculated to conserve the form of the incoming gradient, were used in most experiments (dimensions are given under *Results*). Both ends of the tube were occluded by means of a movable piston (Fig. 2) with a porous glass or Vyon disc (Vyon I, manufactured by Porous Plastics Ltd., Dagenham Docks, Essex, England) which was brought in close contact with the adsorbent. The width of the central channel (Fig. 2) as well as the inner diameter of connecting polyvinyl tubings did not exceed 2 mm.

The gradients were produced with an open cylindrical mixing chamber and a conical reservoir (40, 41). Three sets with different volumes were used according to the size of the column. All gradients in the following are described by two pairs of numbers, as follows: $a \rightarrow b$, $(c + d)$; a is the buffer concentration in the mixing chamber (equal to the initial concentration), b is the buffer concentration in the reservoir; c and d are the corresponding volumes, respectively.

The effluent gradient was checked either by chemical analysis of the eluting ion according to (42) or by determining the conductivity ($\varkappa$) using

the Philips bridges G-M 4144 or PR9501. All chromatography was performed at cold room temperature (about +4°C).

Results

a) *Catalytic ceruloplasmin determination*

Fig. 3 is a survey of several consecutive determinations on the same ceruloplasmin standard and copper standard. The ceruloplasmin standard was a 1 : 101 dilution of Kabi 831 Dtx 28 containing 0.15 *M* NaCl, and 10 μl and 25 μl aliquots were measured. The copper standard was 0.001 *M* cupric chloride of which 100 μl aliquots were used. The general procedure was always as described in *Methods*, but the composition of stock solution I for series A and B was prepared on day 1 (Fig. 3) and consisted of 0.145 *M* DPP · HCl in 2 *N* HCl. This corresponds to a chloride concentration of 8.6×10^{-3} *M* in the final substrate mixture. A part to be used for series B was immediately stored at —20°C, and the solution for the A series was kept in a refrigerator. Similarly, the solution for series C and D was prepared on the 38th day and treated as A and B, respectively. This solution was 0.145 *M* DPP · 2 HCl in 2 *N* HCl, corresponding to a final chloride concentration of 9.2×10^{-3} *M*. The final pH at +37°C of the substrate mixtures in these experiments was about 5.3. Fig. 3 shows that the blank values increased successively when the stock solutions I were stored non-frozen. The solution prepared from the dihydrochloride gave lower initial blank values. Freezing of the solution prevented the blank absorbancy increment. At the same time as the increased blank values there was a decrease of the degree of colouration produced through the activity of the ceruloplasmin standard, while the catalytic effect of the copper standard remained largely unaffected. Disregarding the slope of the line through the points representing the ceruloplasmin standard, the reproducibility may be estimated as within ± 5 per cent. The ratio of the absorbancy generated by 10 μl to that from 25 μl aliquots was usually slightly larger than 2 : 5.

Fig. 4 demonstrates the relation between ceruloplasmin concentration and catalytic formation of the coloured product. Here the standard procedure was used (see *Methods*), and various dilutions with 0.25 *M* potassium phosphate buffer, pH 6.8, of a purified ceruloplasmin preparation (Kabi 1219, DwZ 134) with known absorbancy at 610 mμ, served as the standard. The tubes corresponding to curve B contained 25 μl of the ceruloplasmin dilutions, 100 μl of 0.25 *M* potassium phosphate buffer, pH 6.8, and 2 ml of the substrate mixture. The tubes corresponding to curve A contained 25 μl of the ceruloplasmin dilutions, 100 μl of serum, which had been dialysed against 0.25 *M* potassium phosphate buffer, pH 6.8, and 2 ml of the substrate mixture. The displacement of curve A is due to the ceruloplasmin of added serum. The concentration of ceruloplasmin

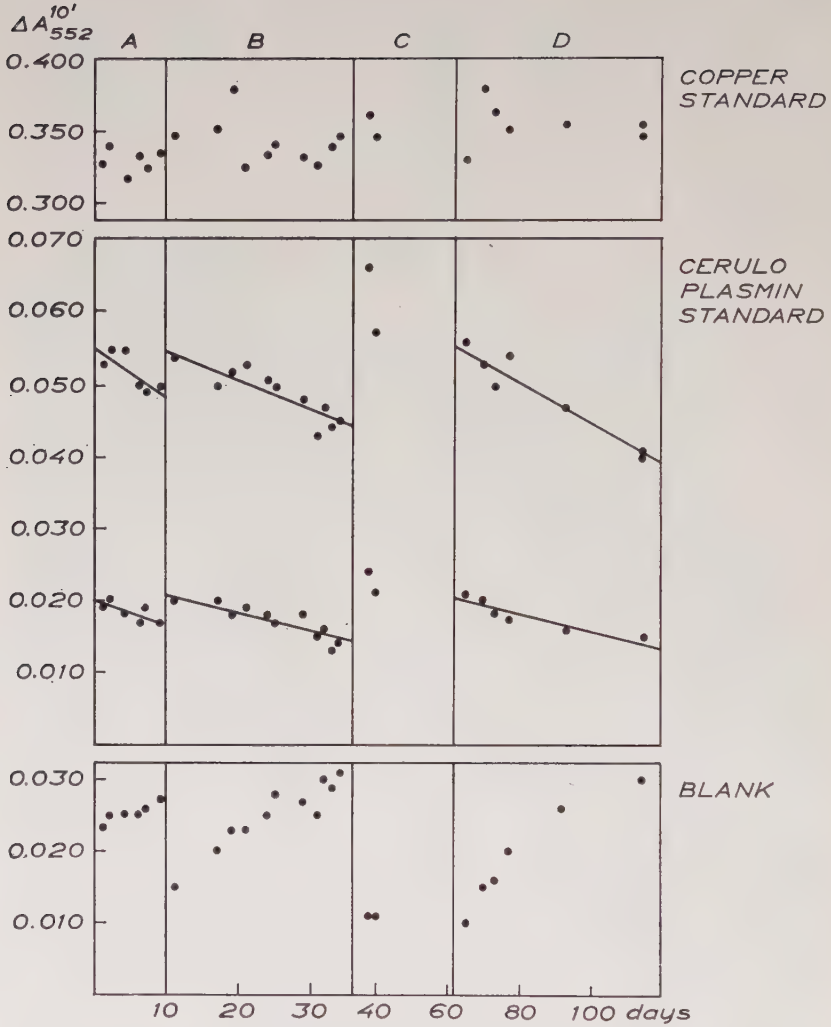

Fig. 3. Survey of blank and standard values in several consecutive series of spectrophotometric ceruloplasmin determinations. With DPP as substrate 10 μl and 25 μl of the same ceruloplasmin standard and 100 μl of the same $CuCl_2$ standard were used throughout. Stock solutions for A and B (0.145 *M* DPP · HCl in 2 *N* HCl) and for C and D (0.145 *M* DPP · 2 HCl in 2 *N* HCl) were prepared on day 1 and 38 respectively. The portions for B and D were stored frozen until required.

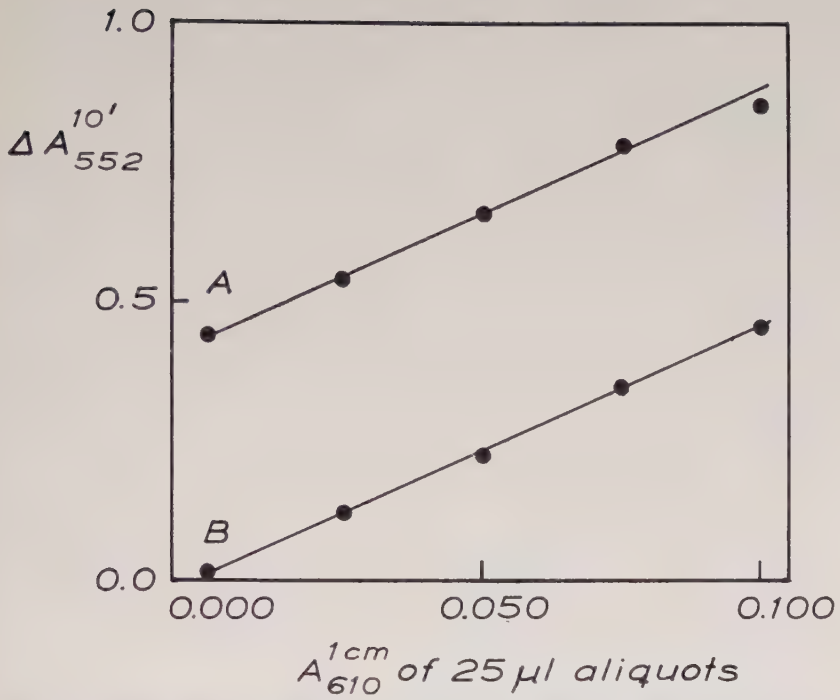

Fig. 4. Relation between ceruloplasmin content (abscissa) and oxidase activity (ordinate) according to standard method. Curve B with 0.1 ml of buffer and curve A with 0.1 ml of serum added to 2 ml of the substrate mixture.

copper in this serum can be calculated from Fig. 4 and the formula given by Holmberg and Laurell (4): $(25/100)\times 5\times 0.1 = 0.125$ mg/100 ml. The 552 mμ absorbancy range of these samples corresponds to the range of routine measurements.

Fig. 5 shows the efficiency of the ice-bath as a means of interrupting the incubation. The outcome of 15 consecutive measurements is represented (corresponding to A and B of Fig. 3). Two tubes with 25 μl of the ceruloplasmin standard (see Fig. 3 and above) were measured, the first one within 10 minutes after putting the tubes in the ice bath, the second one after an additional interval as indicated by the x-axis in Fig. 5. The absorbancy of the second standard is expressed as per cent of the absorbancy of the first standard. The average increment of the absorbancy after 60 minutes in the ice-bath is estimated to be about 5 per cent.

Figure 6 shows the effect of EDTA. This was included in the substrate mixture (final concentration $4\times 10^{-5}\ M$) in order to suppress the catalytic activity of contaminating metal ions, particularly cupric ions (cf. the copper standard in Fig. 3). Copper forms a chelate with EDTA in the molar

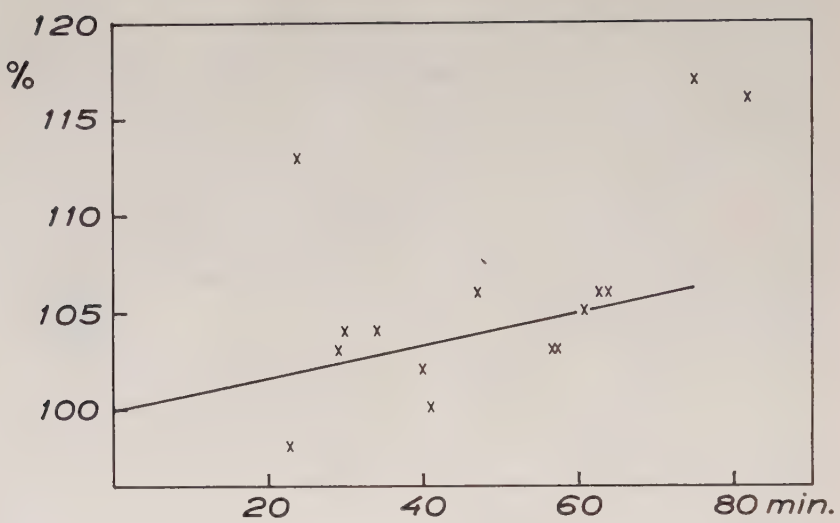

Fig. 5. Effectivity of ice-bath as a means of interrupting the incubation. Results of 15 consecutive series of measurements each including two 25 μl ceruloplasmin standards. Increment of absorbancy of the one standard after various times expressed as per cent of absorbancy of the other standard at time 0.

ratio of 1 : 1, and at pH 3.5 or higher the chelation is almost complete (43). The figure shows that the blank activity (open squares) is practically abolished by concentrations of EDTA higher than about 10^{-6} *M*. This corresponds to the expected level of copper contamination in water and chemicals. That cupric ions do have a relatively intense oxidizing effect on DPP is further demonstrated by the crossed square curve which was obtained with 4.75×10^{-5} *M* cupric chloride added to the blanks (the copper standard in Fig. 3 produced a final copper concentration of 5×10^{-5} *M*). In the experiment represented by Fig. 6 EDTA concentrations higher than the equivalent of the added copper will again suppress the catalytic activity. Thus, the copper EDTA chelate has no ability to catalyze the oxidation of DPP. Experiments with iron indicated that EDTA had no influence whatever on the comparatively moderate oxidation of DPP that was caused by added quantities of ferric chloride. Fig. 6 also demonstrates that the final EDTA concentration used in the standard assay method, 4×10^{-5} *M*, has no inhibitory effect on the oxidation that is catalyzed by ceruloplasmin (closed circles).

b) *Chromatographic experiments*

In order to select a suitable gradient for optimal separation of serum proteins with DPP-activity a preliminary chromatography was performed in which the gradient was extremely extended so as to embrace as many

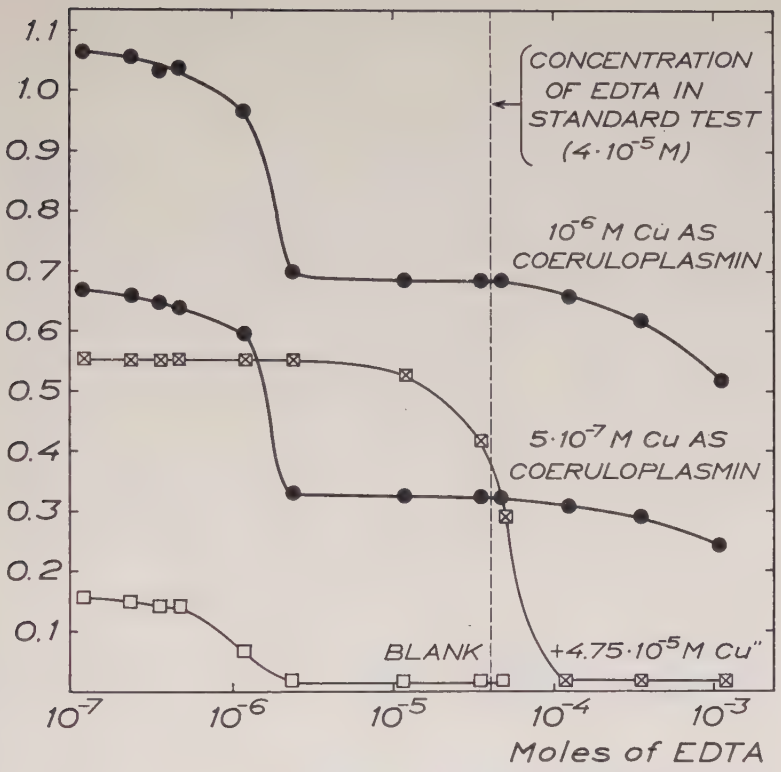

Fig. 6. Effect of different concentrations of EDTA on the oxidation of DPP. Open squares: blank with no additions. Crossed squares: blank with $4.75 \times 10^{-5} M$ cupric chloride added. Filled circles: in the presence of two different concentrations of ceruloplasmin (25).

of the serum components as possible. Fig. 7 shows the result of this exploratory chromatography. The dimensions of the column were 15 cm $\times$ $\times$ 1.5 cm, and 12 ml of serum was applied. The development was carried out with 2 consecutive gradients: (a) 0.005 $\rightarrow$0.15 *M* potassium phosphate, pH 6.8, with 5% sucrose (78 + 283 ml); (b) 0.15 $\rightarrow$0.65 *M* potassium phosphate, pH 6.8, with 5% sucrose (78 + 283 ml). The shift between the 2 gradients occurred near fraction 180. The presence of sucrose in the buffers increases the solubility of globular proteins at the low ionic strength used in the beginning of the chromatogram. Fig. 7 shows that the protein (open circles) has been distributed over a considerable number of fractions and that the catalytic activity (filled circles) is confined to the latter part of the chromatogram. The activity is resolved into two distinctly separated zones, referred to in the following as peak I (fractions 130 –

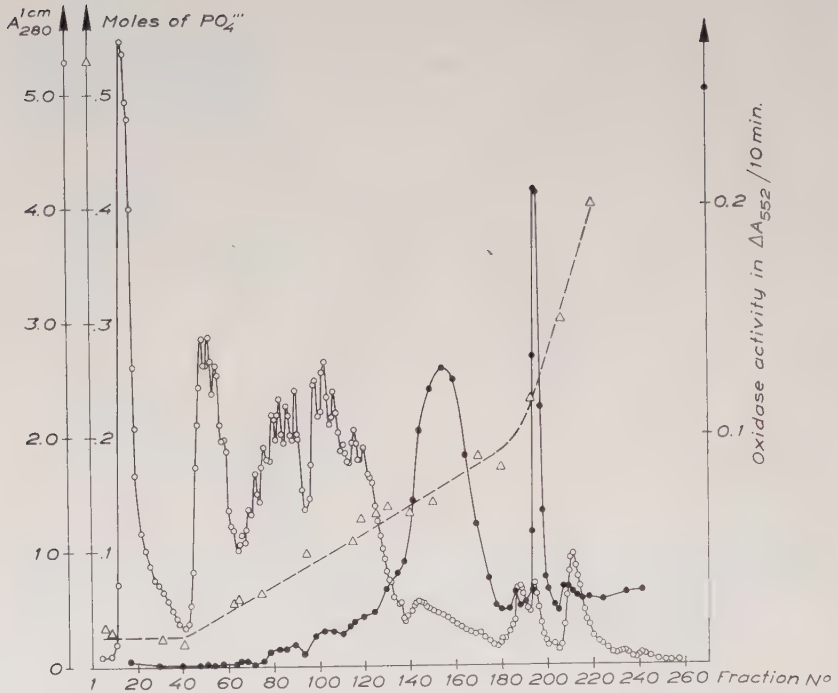

Fig. 7. Gradient chromatography of 12 ml of fresh human serum on a 15 cm × 1.5 cm hydroxylapatite column. Gradient (a): 0.005 → 0.15 *M* pottassium phosphate, pH 6.8 (78 + 283 ml); (b): 0.15 → 0.65 *M* potassium phosphate, pH 6.8 (78 + 283 ml). All buffers contained 5% sucrose. Catalytic activity: 200 μl aliquots. Open circles: absorbancy at 280 mμ, filled circles: catalytic activity, triangles: phosphate concentration (26).

180) and peak II (fractions 192—203). In this figure, no corrections have been made for the auto-oxidation of the substrate which is responsible for the slope of the baseline starting around fraction 70. No catalytic effect due to serum constituents is detectable at lower phosphate concentrations than 0.125 *M*, corresponding to fraction 130, and this concentration was chosen for the starting buffer in most subsequent chromatographies. A typical example is shown in Fig. 8. In this experiment 9 ml of serum was chromatographed on a 1.3 × 13.5 cm column (about 4 g dry weight of the adsorbent) with the gradient 0.125 → 0.66 *M* (78 + 283 ml). With this relatively concentrated starting buffer most serum proteins pass the column without being adsorbed and appear as a large peak immediately after the void volume (fractions 9—16). No activity escaped with this component. The two catalytically active peaks, with maxima in fractions 54 and 66 respectively, showed the same visible spectrum with maximum

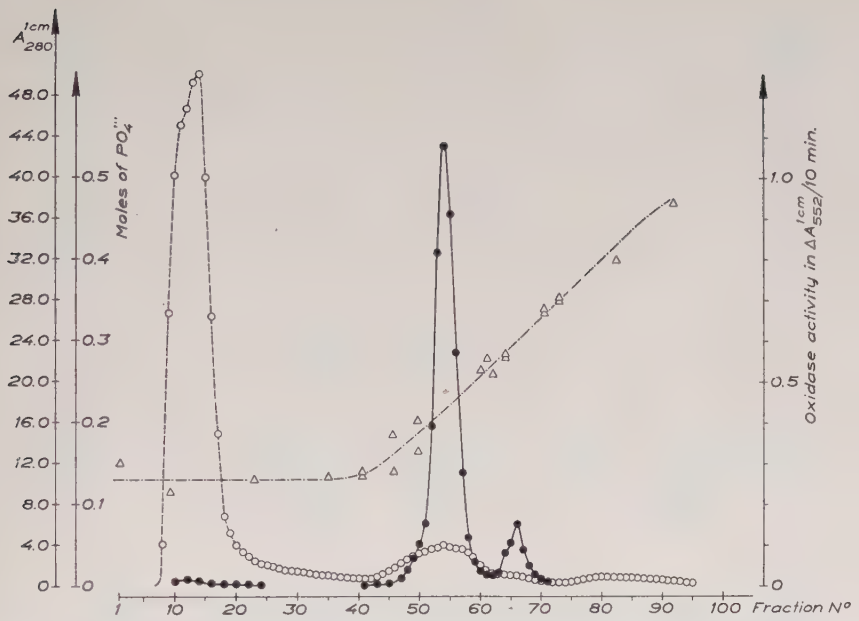

Fig. 8. Gradient chromatography of 9 ml of fresh human serum on a 13.5 × 1.3 cm hydroxylapatite column. Gradient: 0.125 →0.66 *M* potassium phosphate buffer, pH 6.8 (78 + 283 ml). Open circles: absorbancy at 280 mμ, filled circles: catalytic activity (100 μl aliquots), triangles: phosphate concentration (25).

absorbancy at 605—610 mμ, characteristic of ceruloplasmin (4). Fig. 9 illustrates the corresponding separation on a larger scale. In this experiment 437 ml of retroplacental serum was chromatographed on a 4.4 × 94 cm hydroxylapatite column with the gradient 0.125 →0.50 *M* (800 + 5320 ml). Only the part of the chromatogram is shown that corresponds to fractions 35 through 95 in Fig. 8.

The ratios of the sum of activity times fraction volume for peak I to that of peak II varied between 3 : 1 and 6 : 1 (cf. Chapter 2). The recovery of active material in the type of chromatography described in this chapter was about 70%.

With regard to the original observation by Uriel *et al.*, which was described in the *Introduction*, it was of interest to see how purified ceruloplasmin preparations behave in the hydroxylapatite gradient system. A typical result is demonstrated in Fig. 10, (only the part under the gradient is shown). The starting material was 1.5 g of Kabi 1219 DwZ 131, and the chromatography was performed on a 85 × 4.4 cm column with the gradient 0.125 →0.50 *M* (800 + 5320 ml). Three peaks were obtained under the gradient (maxima at fractions 195, 235 and 295, respectively)

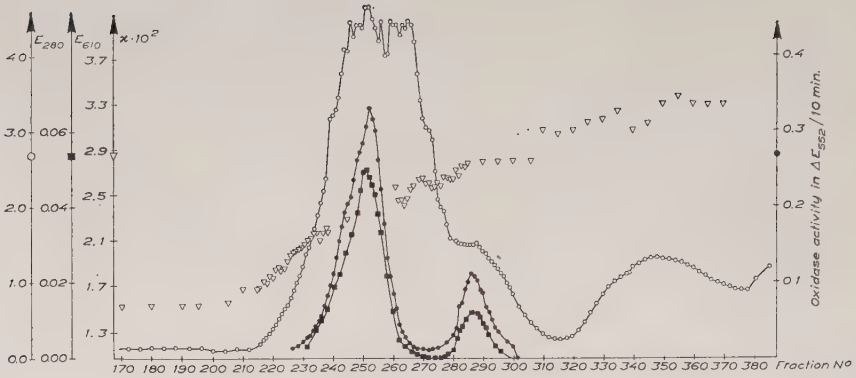

Fig. 9. Gradient chromatography of 437 ml of pooled human retroplacental serum (non-frozen) on a 94×4.4 cm hydroxylapatite-Pevicon column. Gradient 0.125 → →0.50 *M* potassium phosphate buffer, pH 6.8 (800+5320 ml). Open circles: absorbancy at 280 mμ, filled circles: catalytic activity (25 μl aliquots). Filled squares: absorbancy at 610 mμ, triangles: conductivity (ϰ) (26).

with rather good conformity of the absorbancies at 280 mμ and 610 mμ. Several chromatographies of various Kabi preparations as well as one Squibb preparation all contained three ceruloplasmin components (and varying amounts of contaminating proteins).

Discussion of the ceruloplasmin assay method

The concentration of a catalyst can be assayed through determination of the rate of disappearance of a suitable substrate or the rate of formation of a characteristic product of the catalyzed reaction. Complications occur, however, if the measured species is lost or formed in secondary reactions and if a product disturbs the activity of the catalyst. For the present investigation an assay reaction was needed that 1) does not involve serious complications of this kind, and in addition 2) is specific for ceruloplasmin in the treated material, and 3) is suitable for determinations in a large number of chromatographic fractions at the same time.

Spectrophotometric determinations are comparatively accurate and rapid. Fortunately, the catalyst itself, as well as several of its substrates, reacts with conspicious changes of absorbancy at unique wavelengths, thus offering a large choice for various experimental situations.

p-phenylenediamine (PPD) and its *N*-methyl derivatives form strongly coloured oxidation products. In the first oxidation step they lose one electron per molecule, and a more or less stable free radical appears. The unsubstituted form is the best substrate for ceruloplasmin but its first oxidation stage has a comparatively short half-life (44, cf. also 29). The *N*,*N*-

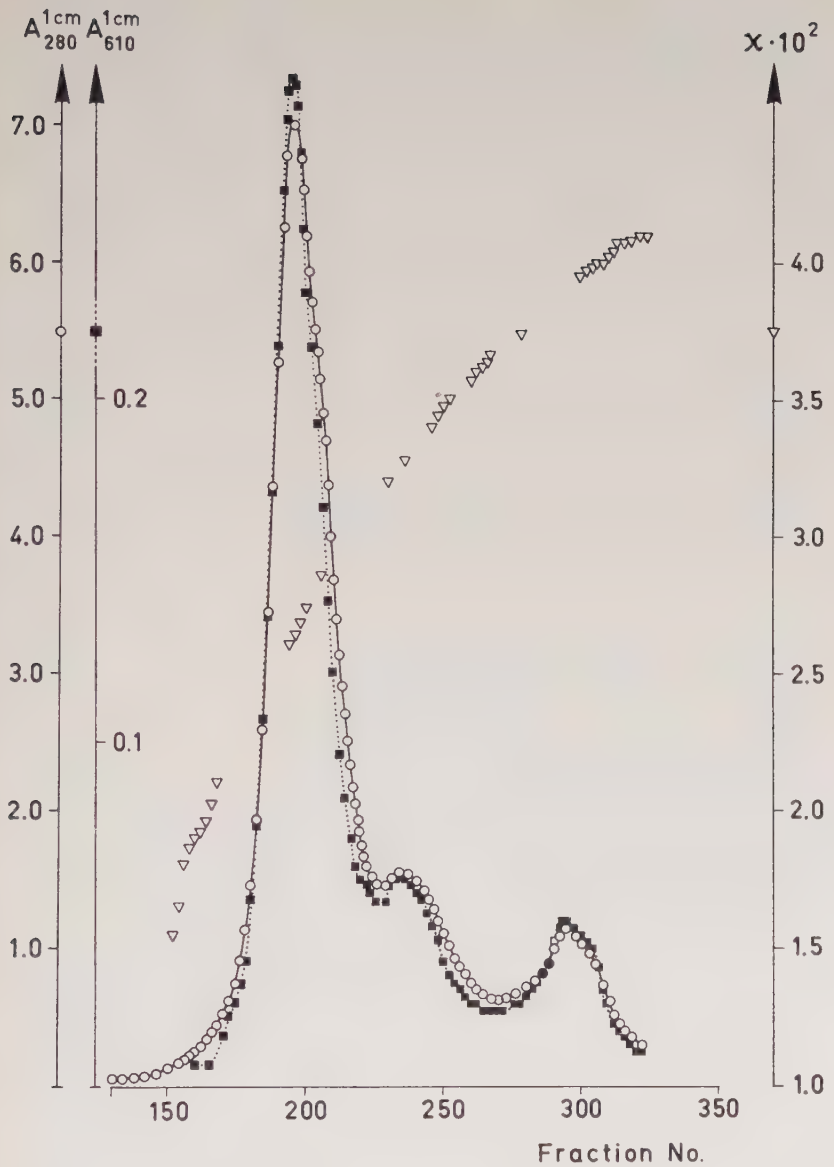

Fig. 10. Gradient chromatography of 1.5 g of purified ceruloplasmin (Kabi 1219 DwZ 131) on a 85×4.4 cm column with the gradient 0.125 →0.50 *M* potassium phosphate buffer, pH 6.8 (800+5320 ml). Open circles: absorbancy at 280 mμ, filled squares: absorbancy at 610 mμ, triangles: conductivity ($\varkappa$).

dimethyl (DPP) and the tetramethyl (TPP) derivative, on the other hand, form first oxidation products that are unusually stable in water under certain conditions (45). These free radical or semiquinoid forms are called Wurster's red and Wurster's blue, respectively. The semiquinoids have strong absorption bands in the visible region that are totally absent in the leuco forms, for example, at 552 mμ for Wurster's red, and at 560 mμ for Wurster's blue. DPP was preferred in the present case, because its autooxidation is more effectively controllable than that of TPP. The pH used for the substrate mixture (5.1 at the incubation temperature, +37°C) was a compromise between the optimal pH for ceruloplasmin activity in acetate buffers, between 5 and 6 (7), and the condition that gives maximal stability of the free radical, pH = 4.6 (45). Though uncommonly long for a free radical the half-life of Wurster's red is nonetheless limited, and a short incubation interval (10 minutes) at an elevated temperature seems preferable to a prolonged incubation at room temperature. Another reason for this policy is the indication in Fig. 3 that some not identified product that inhibits the catalyst may be formed at a slow rate. Obviously, this can not be the free radical itself as evidenced by the linearity over a wide range of first product concentrations (Fig. 4). Peisach and Levine (44) observed an inhibitory effect of—probably secondary—products formed shortly after ceruloplasmin-catalyzed oxidation of PPD, whereas the comparatively stable first oxidation product (Wurster's blue) formed during oxidation of TPP did not display any inhibitory effect. In order to counteract the formation of the inhibitory product during storage of stock solution I, the HCl concentration was increased to 4 *N* and the storage temperature lowered, (—20°C) beneath the freezing point. The final DPP concentration, $5.8 \times \times 10^{-4}$ *M*, which is lower than the one needed to make the catalyst-substrate formation a zero order reaction, was maintained for the same reason. See the curve in Fig. 11, which was obtained under the conditions of the standard method (freshly prepared substrate solution) with the exception that initial rates were measured (see *Methods,* Chapter 4). K_m calculated from the curve is 10^{-3} *M*. These measures were sufficient to eliminate the increase of the blank values but they were not wholly effective in counteracting the formation of the inhibitory species. In spite of this the method was deemed satisfactory for the intended purpose which was to determine the relative concentrations of ceruloplasmin in starting material and chromatographic fractions. Fig. 4 shows that the linearity of the concentration —activity relationship is not disturbed by the presence of plasma proteins.

Determinations of the absolute concentration of ceruloplasmin, for example in serum samples, can be done with the method described here if a suitable standard is included (Fig. 4).

It is desirable to use the same final concentration of chloride in reaction mixtures that are to be compared. Holmberg and Laurell (7) showed that the rate of PPD oxidation by ceruloplasmin is dependent on the chloride concentration and that an optimal concentration exists, different for different pH-values. The nature of this effect is uncertain, on the one hand

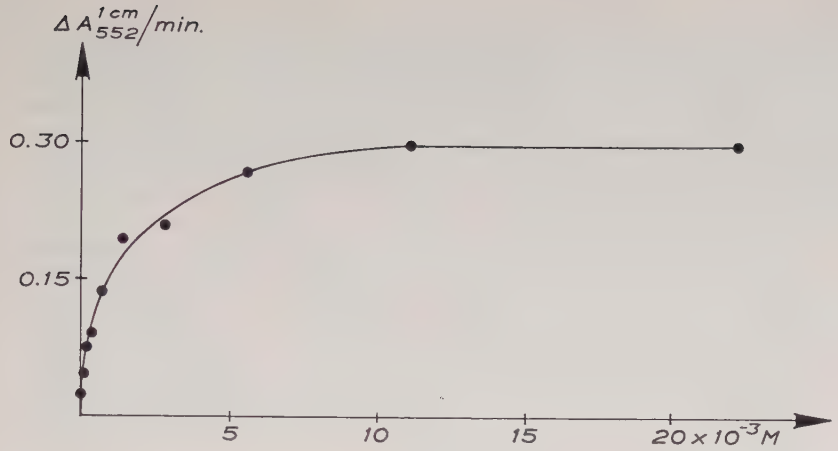

Fig. 11. Dependence of the initial rates of formation of Wurster's red (ordinate) on the concentration of DPP (abscissa) with a constant amount of ceruloplasmin (25 μl of Kabi 1219, DwZ 134). Cf. *Methods,* Chapter 4. Other ingredients of substrate mixture, and temperature as for standard method.

chloride ions appear to be necessary for maintaining the stability of peak I ceruloplasmin (see Chapter 2), on the other hand there are kinetic observations indicating that the stability of the PPD radical depends on the chloride ion concentration (29). The author found an optimal chloride concentration of about 8 m*M* for the ceruloplasmin-catalyzed oxidation of TPP at pH 5.3. Halving this concentration caused a decrease of the apparent initial oxidation rate of about 2 per cent, and doubling the chloride concentration caused a decrease of about 7 per cent. Corresponding data for the oxidation of DPP are unfortunately not available, but an effect of the difference in chloride content is suggested as a possible explanation of the deviation from linear proportionality between absorbancies caused by 10 μl and 25 μl aliquots of the ceruloplasmin standard in Fig. 3.

Simultaneous treatment of as many samples as 84, and the relatively short incubation period used, made interruption of the catalysis by pipetting an inhibitor such as cyanide or azide into each test tube impracticable. However, lowering of the temperature was sufficiently effective for the present purpose (Fig. 5). It should be noted that no corrections have been made for the continued activity of samples in the ice-bath during the reading in any of the chromatograms.

The role of EDTA in ceruloplasmin-catalyzed reactions has arisen considerable interest in many quarters (46—53). This question is still somewhat controversial and involves such problems as the effects on ceruloplasmin of anions in general (7) and of polycarboxylic acids in particular (54). EDTA and other chelating agents may on the other hand only in-

directly affect the oxidation catalyzed by ceruloplasmin, either as a stimulator by binding inhibiting metal ions, or as an inhibitor by binding certain transition metal ions that may form reduction-oxidation couples between ceruloplasmin and substrate (49). However, there is little doubt that EDTA also suppresses the independent catalytic activity of contaminating cupric ions (Fig. 6, cf. the copper standard in Fig. 3). The amount necessary for this purpose does not affect the ceruloplasmin activity appreciably (Fig. 6).

The assay method outlined here may be characterized as specific for ceruloplasmin among serum proteins, with the proviso that a very moderate activity of an uncertain nature may be observed corresponding to the last protein peak which consists largely of β-lipoproteins (37). Hemolytic sera do not show any additional activity peaks, but corrections should be made for the absorbancy at 552 mμ caused by the haemoglobin (Fig. 24, page 41). Examples of the reproducibility of the assay method in its practical application are given in Fig. 26 page 43.

Discussion of the chromatographic method

Works by Tiselius, Hjertén and Levin (36, 37, 38, 55, 56) have largely contributed to the improvement of calcium phosphate chromatography as a tool in protein chemistry. A number of purified proteins were used as model substances in these methodological investigations. Of particular interest for the present problem is the observation that multiple components were obtained from several presumedly homogenous proteins (36), suggesting an uncommonly high resolving power of this system. Furthermore, investigations on whole serum and on purified ceruloplasmin (37) showed that hydroxylapatite has a relatively high capacity for this protein and effectively separates it from the bulk of serum proteins. However, there was no comment on the heterogeneity of ceruloplasmin in these investigations.

One naturally asks whether the observed heterogeneity of serum ceruloplasmin (Figs. 7, 8 and 9) is artificial or physiological. The third peak occurring in purified ceruloplasmin (Fig. 10) is obviously an artifact, since chromatograms of a rather large number of fresh human sera carried out by the author with a variety of gradients and amounts of starting material all disclosed only two components.

In the present chapter, I shall limit the discussion to the question of whether the chromatographic procedure itself may be responsible for a spurious subdivision of the protein. Artifacts of this kind are not unknown. Boman (41) lists 7 conditions that can produce double zoning in protein chromatography. All but one of these can, however, be excluded in the present case: 1) overloading of the column with respect to ceruloplasmin would cause activity to appear in the well separated first peak (Fig. 8, fractions 9–16); 2) zones with abnormal pH did not occur with standard operation, according to several controls; a related type of artifact can be produced if the starting material is applied in NaCl-containing

buffer with which the column was not equilibrated (Fig. 12); 3) the typical multiplication of peaks that may be observed in stepwise elution is ruled out, because gradient elution was employed; the form of the outcoming gradient was analyzed in a multitude of chromatographies with various column sizes and different relative loads, and the same steady slope over the ceruloplasmin-containing part as in Figs. 8—10 was always obtained; slow fluctuations of the pH, not exceeding two tenths of a unit were, however, observed (cf. ref. 38); 4) the heterogeneity is not due to denaturation or similar changes during the chromatographic process, because each ceruloplasmin peak was homogeneous on re-chromatography in the same system (see next chapter); 5) effects that may be caused by change of buffer ions were not to be expected under standard conditions (cf. Fig. 12); 6) the same is probably true, with the pH used, for disturbances that may be caused by dissolved carbon dioxide of the air; 7) the possibility of complex formation with proteins or small molecules borders on the general question of functional or artificial changes of the ceruloplasmin molecule and is more conveniently discussed in the succeeding chapter.

Another indication that the three peaks from purified ceruloplasmin appearing under the gradient (Fig. 10) are due to as many distinct forms of ceruloplasmin with different affinities for the adsorbent is provided by the observation that three blue adsorption zones are formed by protein-protein displacement in the presence of starting buffer (Fig. 13). Cf. also the different mobility in starch-gel electroforesis of material from different hydroxylapatite peaks observed by Hirschman *et al.* (ref. 67 and next chapter).

The outflowing concentration gradient, as well as the pH of fractions, was traced mainly to make sure that an observed heterogeneity was not due to step formations or other irregularities. Chemical phosphate determinations were not very accurate in our hands and were soon abandoned. Thus, the gradient in Fig. 7 is somewhat dislocated upwards giving a too high starting buffer concentration. The choice of starting concentration for subsequent chromatograms was nevertheless successful (Fig. 8). If a number of variables, such as temperature and pH, are kept constant, there is for each protein a critical buffer concentration at which comparatively small increments of the ionic strength causes a large increment of the R_f value (56). This circumstance would provide a convenient means in gradient chromatography of establishing the identity of a protein by determining the buffer concentration at which it appears in the eluate. There are, however, two important factors that can cause a protein to leave the column before its R_f has reached unity: 1) If the slope of the gradient is small, *i.e.* if the buffer volume needed to change the ionic strength by one unit at a given level of the column is large, the protein will travel a comparatively long distance before unit R_f is reached. This means that if the column is short compared to this distance the protein will leave the column at an unknown R_f value. 2) The second and more important

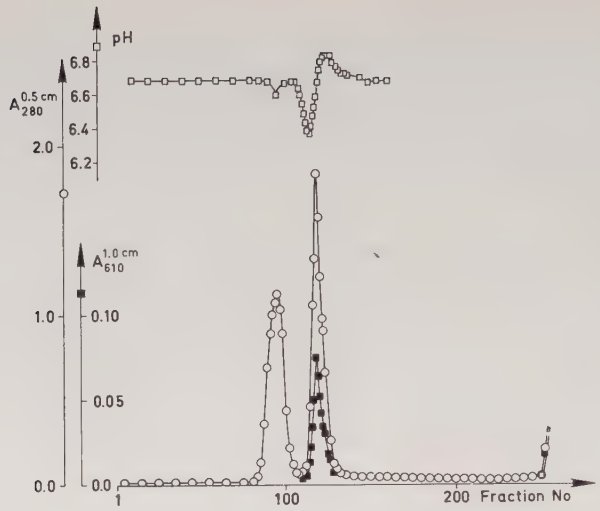

Fig. 12. Chromatographic artifact (peak with maximum in fraction 118) produced by applying the starting material (1 g of Kabi 1219 DwZ 114) dissolved in 0.15 *M* NaCl + 0.125 *M* potassium phosphate buffer, pH 6.8. The 81 × 4.4 cm column was equilibrated with 0.125 *M* potassium phosphate buffer pH 6.8, and the sample was followed by additional buffer of this composition. Open circles: absorbancy at 280 mμ, filled squares: absorbancy at 610 mμ. Open squares: pH.

complication is due to the fact that a strongly adsorbed protein will displace a less strongly adsorbed protein and that the height of an adsorption zone will increase with increasing ionic strength. This means that a protein may be displaced out of the column by expanding zones behind it before

Table 1. *Phosphate concentrations at which the ceruloplasmins in the chromatograms of Fig. 8, 9, and 10 appeared in the eluate.*

Fig.	Peak with maximum in fraction	Notation	Concentration of eluting buffer, *M*	Conductivity ($\varkappa$) of eluting buffer, 10^{-2} ohm^{-1} cm^{-1}
8	54	I	0.22	
	66	II	0.30	
9	251	I	0.21[a]	2.4
	287	II	0.24[a]	2.8
10	195	I	0.23[a]	2.6
	235	IIa	0.30[a]	3.3
	295	IIb	0.37[a]	3.9

[a] Calculated from the conductivity value.

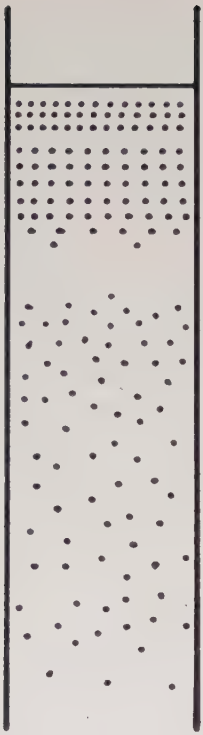

Fig. 13. Adsorption zones on a 68 × 1.9 cm hydroxylapatite column 26 hours after applying 0.5 ml of purified ceruloplasmin (Kabi DwZ 67) followed by 0.125 *M* potassium phosphate buffer, pH 6.8. Relative densities, heights and degree of demarcation are indicated.

the critical ionic strength is reached. The difficulties may be overcome by using long columns and moderate loads when the sample contains proteins that are more strongly adsorbed than the species of interest. In the case of serum, β-lipoproteins, among others, will displace the ceruloplasmin.

Elution data from the chromatograms in Figs. 8—10 are collected in Table 1. The effect of displacing proteins is visible in the chromatography in Fig. 9 where the load was comparatively heavy. The position of peak II is particularly affected, as can be seen when compared to the position of peak II in Fig. 8. Eluting concentrations for the three peaks obtained from purified ceruloplasmin (Fig. 10) should be more reliable as true characteristics of the components. For the reasons mentioned it is thus not possible to conclude directly from the positional data of Table 1 which one, if any, of peaks IIa and IIb in the purified preparation is identical with peak II of fresh serum.

CHAPTER 2

THE HETEROGENEITY OF HUMAN CERULOPLASMIN

Introduction

In recent years, the existence in individual organisms of minor structural differences in proteins, formerly considered homogeneous, has become apparent through improved methods of analysis. These variants may, for example, be recognized by their different behaviour in one or several separation systems. The structural deviation may affect certain physico-chemical parameters or a specific function, as defined by some *in vitro* reaction. Absence of functional differences between structural variants does not necessarily imply that they are functionally equivalent *in vivo,* since an *in vitro* reaction often brings into play only a section of the repertory of the macromolecule. Ultimately, the structural multiplicity is due to the existence of separate genetic loci that control the biosynthesis of the molecule. This diversification is the result of duplications and mutations that once caused limited alterations in the DNA of the chromosome. Different structural variants of a protein may be exclusively or predominantly produced during alternating developmental periods, or they may be synthesized simultaneously but in different tissues. Furthermore, the relative proportions of variants may differ in different populations of organisms. Some mutations cause more or less impaired *in vivo* function of the corresponding protein variant ("molecular disease"), but such a functional impairment *in vivo* may not be apparent in an *in vitro* test.

Clearly, true structural variants of proteins are important objects of investigation, from a practical point of view as well as theoretically. On the one hand, they can contribute to the classification and localization of disease. On the other hand, they are of interest in such fields as genetics, embryology, comparative physiology, enzymology, and metabolic control. Therefore, a crucial step in the analysis of an observed heterogeneity is to make certain that the components really represent true structural variants. The surest way to do this, is, of course, to analyse the different species for general conformation, for amino acid composition and sequence, and, in the case of ceruloplasmin, also with respect to the binding modes, the valency states and the number of copper ions, and the nature and content of carbohydrate units. Quite apart from the vastness of such a project, a prerequisite would be that the various forms are available in a pure state, and that the purification methods employed do not induce structural changes. With purification techniques available to-day these two conditions may, in fact, be incompatible.

It is, however, to some extent possible to infer about the nature of a heterogeneity from indirect observations. Thus, a truly genetic heterogeneity is probable if it can be shown that the relative amounts of the variants are different in different populations, at different developmental stages or in hereditary diseases that involve the biosynthesis of the protein in question. Conversely, a true structural difference is ruled out if it can be shown that the observed heterogeneity can be produced by some functional alteration of the protein or is a result of the preparation procedure.

In the preceding chapter artifacts due to the chromatographic method were discussed. It seems practical for the present problem to distinguish between chromatographic artifacts in a narrow sense and artificial heterogeneity due to other causes, including functional variations of the protein. It was concluded that chromatographic artifacts were probably not at the bottom of the multiplicity of human ceruloplasmin, *i.e.* there were no indications that a homogeneous population of ceruloplasmin molecules were submitted to chromatographic conditions, such as insufficient capacity, allowing only some of the proteins to be adsorbed, or fronts between different buffer ions dividing a homogeneous zone, so that molecules on one side of the front reacted differently from identical molecules on the other side of it (Cf. Fig. 12).

The present chapter describes a different kind of chromatographic artifact obtained with diethylaminoethyl (DEAE) ion exchangers, and also some other conditions that produce auxiliary active components. Other problems investigated are correlations between the heterogeneity of ceruloplasmin and developmental stage as well as presence of Wilson's disease.

The investigation on Wilson sera, here presented for the first time, was carried out in 1958—60, in collaboration with Dr. A. G. Bearn of the Rockefeller Institute, New York, U.S.A.

Materials and Methods

Normal sera, retroplacental serum and purified Kabi ceruloplasmin were obtained and treated as described in Chapter 1.

Sera from Wilson patients were shipped under sterile conditions and frozen in dry ice from New York to Uppsala. An equally treated normal serum was included. Sera from blood obtained from the umbilical cord of newborns were treated as individual sera in Chapter 1. The sera were subsequently pooled and dialyzed as described in that chapter. (I am indebted to Prof. C. Gemzell, the Obstetric Clinic, Roy. Acad. Hosp., Uppsala, for the cord blood sera used in this investigation).

Ceruloplasmin-containing chromatographic fractions were pooled as indicated under *Results* and concentrated according to (76). Dialysis against starting buffer was as for serum samples.

Catalytic and conductometric assay methods and the apatite-chromatographic procedure was as described in the preceding chapter. The diethylaminoethyl-ion exchanger DEAE-Sephadex G-50, 100 200 mesh, which

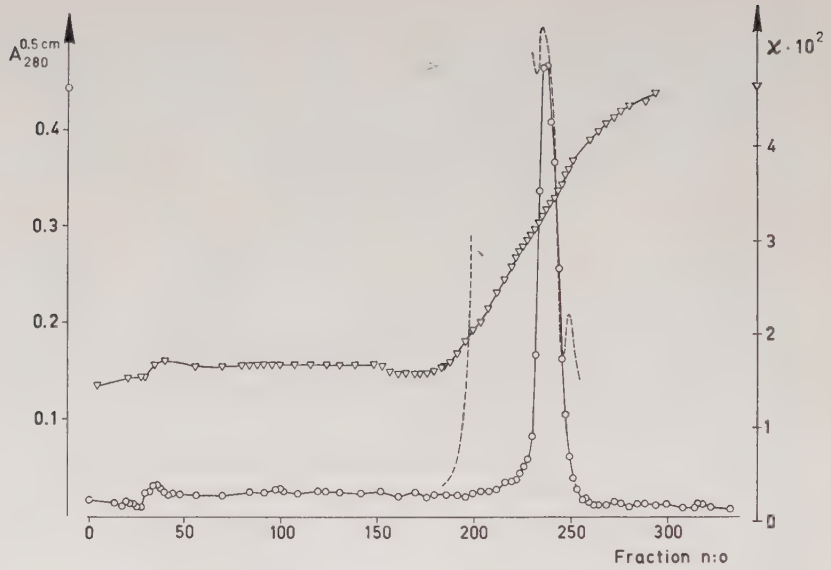

Fig. 14. Re-chromatography on hydroxylapatite of ceruloplasmin from peak IIa. In a preliminary chromatography 1 g of purified ceruloplasmin (Kabi DwZ 114) was run under similar conditions as shown in Fig. 10. The preliminary chromatogram is indicated by the dashed line; the amplitude is arbitrary, and the lateral position of peaks IIa and IIb is such that the conductivity of the eluate is given by the conductivity curve of the re-chromatography (triangles). The starting material for the re-chromatography was 20 ml of the pooled fractions of the right half of peak IIa in the original chromatogram. After dialysis against 0.125 *M* potassium phosphate buffer, pH 6.8, this material was run on a 39×1.5 cm hydroxylapatite column with the gradient 0.125 →0.50 *M* (78+283 ml) potassium phosphate buffer, pH 6.8. Open circles: absorbancy at 280 mμ.

has chromatographic properties similar to DEAE cellulose, was obtained from AB Pharmacia, Uppsala, Sweden. The ion exchanger was pretreated with 1 *N* hydrochloric acid and 0.25 *N* sodium hydroxide, alternating with washings in distilled water. The DEAE-Sephadex was used in chromatographic experiments with the column tubes and gradient mixing device described in Chapter 1. After packing, the column was washed with buffer of the starting concentration until equilibrium was reached. Unlike hydroxylapatite the DEAE-Sephadex is liable to marked volume changes, and the columns were therefore re-packed between runs after regeneration of the adsorbent with 1 *N* hydrochloric acid. The shrinkage of the adsorbent with increasing ionic strength was compensated for by adjustments of the top piston (Fig. 2, p. 10).

Epsilon-aminocaproic acid was provided by AB Kabi, Stockholm, Sweden.

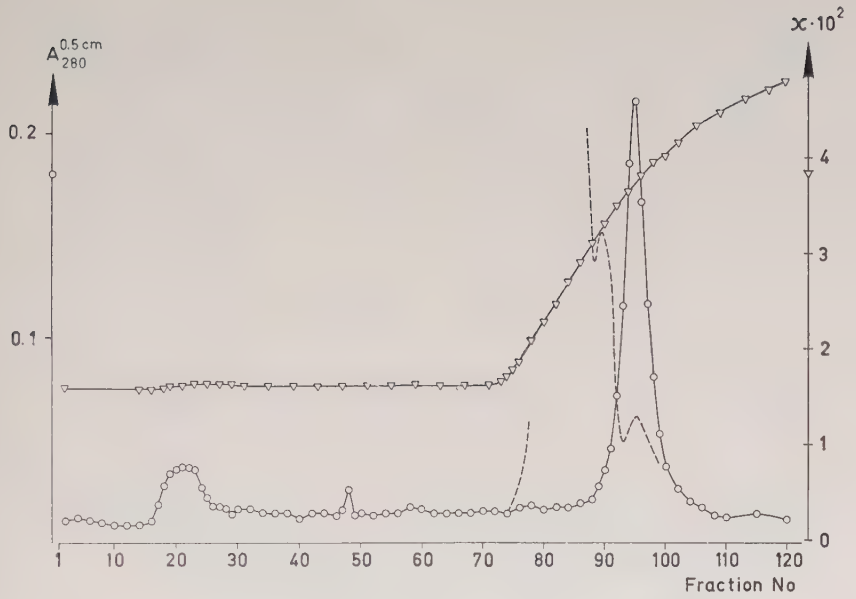

Fig. 15. Re-chromatography on hydroxylapatite of ceruloplasmin from peak IIb. Preliminary chromatography as described in legend of Fig. 14. The starting material was 30 ml of the pooled fractions from peak IIb, of the original chromatogram (dashed curve). After dialysis against 0.125 *M* potassium phosphate buffer, pH 6.8, this material was run on a 39.5×1.5 cm hydroxylapatite column with the gradient 0.125 →0.50 *M* (78 + 283 ml) potassium phosphate buffer, pH 6.8 (open circles). Triangles: conductivity ($\varkappa$).

Non-biological heterogeneity of ceruloplasmin

As mentioned in Chapter 1, re-chromatography of material from the different ceruloplasmin peaks in the hydroxylapatite separation system gave only a single peak in the entire gradient. This is true for the two components obtained from fresh serum (Figs. 7—9) as well as for the three components seen in chromatograms of commercial purified ceruloplasmin (Fig. 10). Figs. 14 and 15 shows re-chromatography of peaks IIa and IIb, respectively (about notations, see Table 1 and Fig. 10). Since these fractions are practically pure, protein-protein displacements can be excluded (see *Discussion*, Chapter 1). The positions in the gradient were reproducible, as can be seen from the superimposed original curve (dashed line). The results show that the heterogeneity can not be due to the chromatographic process, and probably not to the existence in the starting material of an equilibrium mixture of the oxidized and reduced forms of the protein.

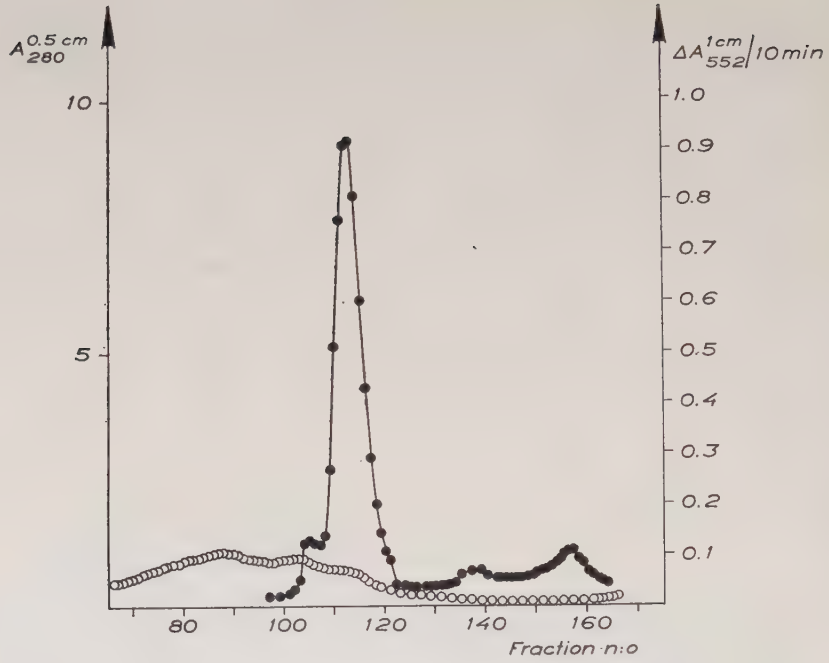

Fig. 16. Effect of storage. Normal, non-haemolytic serum was kept unfrozen in a refrigerator for 17 days before dialysis. 7 ml of the serum was chromatographed on a column containing about 16 g dry weight of hydroxylapatite. Gradient: 0.125 → → 0.50 *M* (78 + 283 ml) potassium phosphate buffer, pH 6.8. Open circles: absorbancy at 280 mμ, filled circles: activity (200 μl aliquots). Only the part corresponding to fractions 40—70 in Fig. 8 is shown.

The third peak in Fig. 10 is, however, definitely not a true structural variant. Its absence in chromatograms of fresh serum indicates that it was formed during some of the preparation procedures used. Various relative mild treatments seem sufficient to bring about this change. Simple ageing of the starting material can cause one or two additional peaks to appear in the chromatogram, as illustrated by the following experiment. A normal, non-hemolytic serum was stored unfrozen in a refrigerator for 17 days before the dialysis. Chromatography of 7 ml of this material (Fig. 16) produced three well separated peaks with maxima in fractions 113, 138 and 157, respectively. In addition, there was a shoulder with maximum in fraction 105. A similar chromatogram was obtained with a serum stored in the same way for 10 days.

Freezing and thawing of the serum before dialysis can also produce a third peak. This is shown in Fig. 17. A fresh serum was divided into two parts. One part, 25 ml, was chromatographed directly (left diagram), and

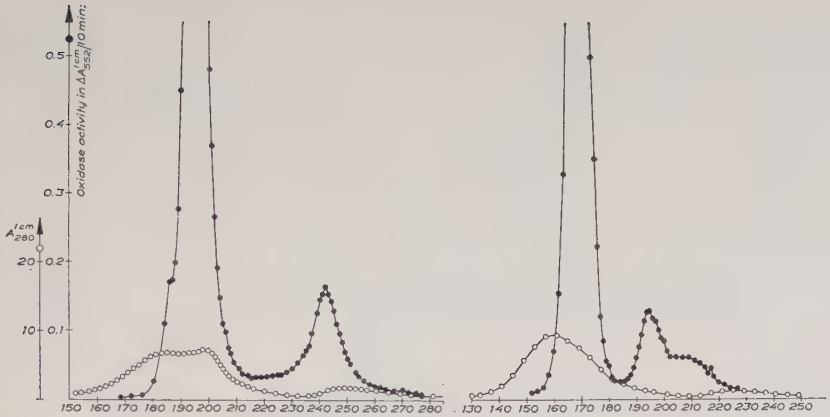

Fig. 17. Chromatograms obtained directly (left curve) and after freezing and thawing (right) of respectively 25 ml and 22 ml of the same fresh normal serum. Column sizes: 27×1.8 cm and 28.5×1.8 cm. Gradient: $0.125 \rightarrow 0.50$ *M* (78 + 283 ml) potassium phosphate buffer, pH 6.8. Open circles: absorbancy at 280 mμ, filled circles: activity (100 μl aliquots). Only the part corresponding to fractions 40—70 in Fig. 8 is shown.

the other part, 22 ml, after freezing and thawing (right diagram). The latter procedure seems to have caused the appearance of two minor, incompletely separated activity peaks.

Unlike the homogeneity shown by all components on re-chromatography in the apatite system, the employment of a DEAE ion exchanger will produce a gradual transformation of the material of the minor peak obtained from fresh serum. This point is demonstrated by the experiments in Figs. 18—20. Pooled fractions 275—300 (peak II) in Fig. 9 (p. 20) were chromatographed on a DEAE-Sephadex column with the gradient $0.10 \rightarrow 0.30$ *M* potassium phosphate buffer, pH 6.8 (Fig. 18). Two approximately equally large activity peaks appeared under the gradient. However, an additional chromatography of each peak (fractions 61—63 and 66—67 respectively) in the same system produced only one active component (Fig. 19) with approximately the same position in the gradient as that of the second activity peak in Fig. 18. Thus, the chromatographic procedure appears to change peak II ceruloplasmin into a variant with increased affinity for DEAE-Sephadex. A corresponding transformation of ceruloplasmin from peak I of Fig. 9 (fractions 238—274) was not observed on chromatography on DEAE-Sephadex (Fig. 27 p. 47). The gradual nature of the transformation of peak II is also indicated by the result of the experiment in Fig. 20, where the starting material comprised about equal parts (according to activity times volume) of ceruloplasmins from peak I of Fig. 9 (once re-chromatographed on hydroxylapatite) and from the two peaks in Fig.

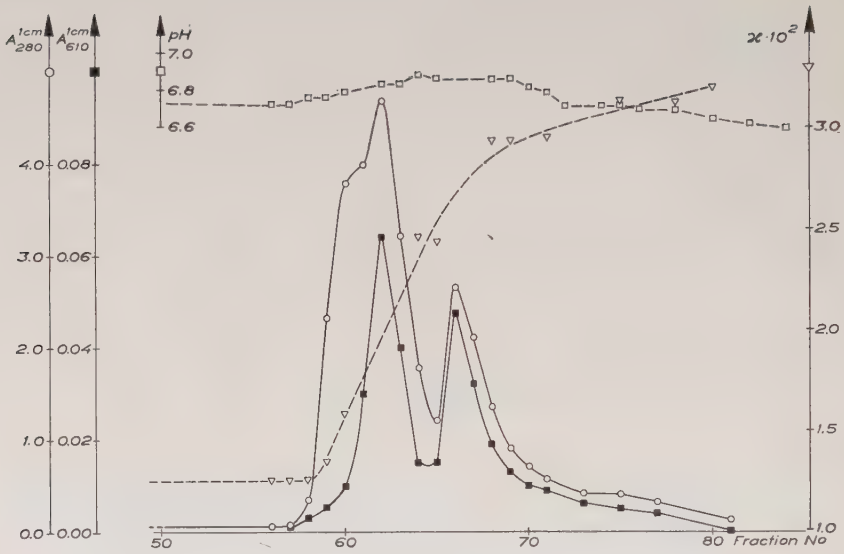

Fig. 18. Gradient elution on a 27.5 × 1.5 cm DEAE-Sephadex column of pooled fractions 275—300 from the chromatography in Fig. 9. Gradient: 0.10 → 0.30 *M* potassium phosphate buffer, pH 6.8 (78 + 283 ml). Open circles: absorbancy at 280 mμ, filled squares: absorbancy at 610 mμ, triangles: conductivity, open squares: pH. Only the part with the gradient is shown (26).

18. Three distinct active components were obtained in the usual apatite chromatogram (Fig. 20). Their positions in the gradient according to the kappa values are about the same as those of the three components respectively in purified Kabi ceruloplasmin (Fig. 10 and Table 1), and their identification with ceruloplasmins I, IIa and IIb seems therefore warranted. The ratios of these peaks (sum of activity times fraction volume), 190 : 300 : 90, were different from the ratios of the pooled ceruloplasmins in the starting material, 240 : 230 : 230. The total recovery of active material was 81%; the recovery of peak I material was 80%; the material of peaks IIa and IIb together comprized 83% of the pooled material from the two peaks in Fig. 18. Thus, the new ceruloplasmin variant which is a product of the DEAE chromatography, appears to have a decreased affinity for hydroxylapatite, though higher than that of peak I which does not seem to receive new material during the transformation.

An observation by Curzon and Vallet (57) may be due to the identical transformation process. These authors obtained two active components from a partially purified ceruloplasmin by a combined concentration and pH gradient chromatography on DEAE-cellulose. One of the components formed a slight but definite peak associated with oxidase activity. Re-

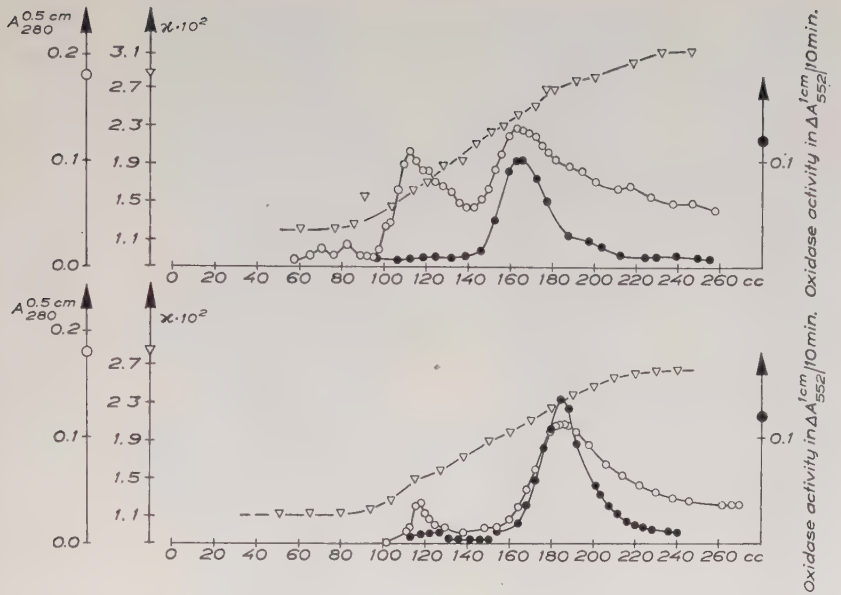

Fig. 19. Gradient chromatographies on DEAE-Sephadex columns with 0.10 →0.30 potassium phosphate buffer, pH 6.8 (78 + 283 ml). *(A) upper curve:* part of pooled fractions 61—63 from Fig. 18. Column size: 23 × 0.9 cm. *(B) lower curve:* part of pooled fractions 66—67 from Fig. 18. Column size: 22.5 × 1.1 cm. Open circles: absorbancy at 280 mμ, filled circles: activity (200 μl aliquots). Triangles: conductivity (26).

chromatography of the major component again produced two active peaks, the minor one containing about 2 3% of the total oxidase activity eluted.

Attempts by the present author to find any conditions that cause the two ceruloplasmins of fresh serum (Fig. 8, p. 19) to be formed from a possible homogeneous original species have failed. Thus, the two active peaks also appeared when plasma was used instead of serum as the starting material. Additions of ε-aminocaproic acid (58) to the blood immediately after tapping (final concentrations about 0.06 *M* and 0.2 *M)* did not abolish the heterogeneity.

Comparison between ceruloplasmins from the three peaks (I, IIa and IIb) obtained on apatite chromatography of purified ceruloplasmin (Fig. 10) did not reveal any significant differences. In the experiment shown in Fig. 21 about 1 g of a purified preparation (Kabi DwZ 131) was chromatographed with the 0.125 →0.50 *M* gradient, and a similar configuration as in Fig. 10 (p. 21) was obtained. The scales in Fig. 21 were chosen so as to give coincident absorbancy curves at wavelengths 610 mμ and

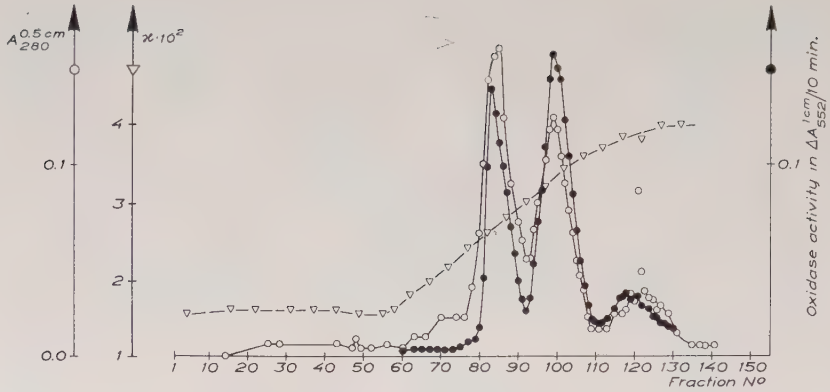

Fig. 20. Gradient chromatography on hydroxylapatite of about equal quantities of ceruloplasmin from peaks I, IIa and IIb (material from respectively fractions 238—260 in Fig. 9, 61—63 and 66—67 in Fig. 18). Gradient: 0.125 → 0.50 *M* (78 + 283 ml), potassium phosphate buffer, pH 6.8. Open circles: absorbancy at 280 mμ, filled circles: activity (200 μl aliquots), triangles: conductivity.

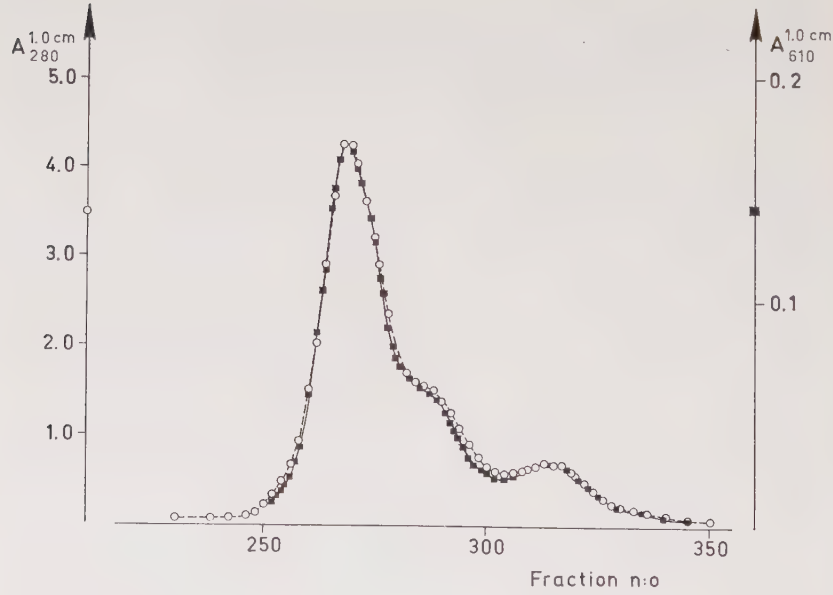

Fig. 21. Gradient elution on a 85 × 4.4 cm hydroxylapatite column of 1 g of Kabi DwZ 131. Gradient: 0.125 → 0.50 *M* (800 + 5320 ml). Open circles: absorbancy at 280 mμ, filled squares: absorbancy at 610 mμ.

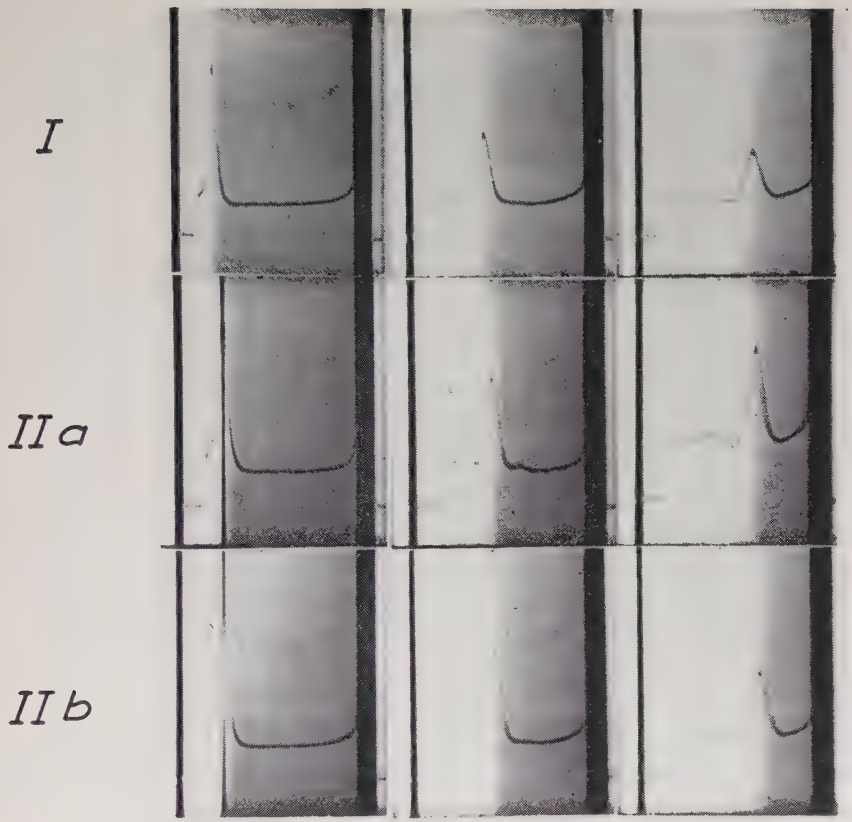

Fig. 22. Ultracentrifugation on a Spinco Model E centrifuge of material from the eluate in Fig. 21. I: fractions 260—278, IIa: fractions 285—300, IIb: fractions 304—335. The pooled material was concentrated according to (76) and dialyzed against 0.1 *M* sodium phosphate buffer, pH 6.8, containing 0.01 *M* sodium chloride. Ceruloplasmin concentration, calculated from the copper concentration (27) and the ceruloplasmin copper content 0.34% (8), was for I: 0.8%, IIa: 0.9%, IIb: 0.9%. Temperature 20°C. Speed: 59,780 rev./min. Exposures for I after 12, 44 and 76 min., for IIa after 16, 46 and 80 min., for IIb after 23. 55 and 87 min.

280 mμ for fractions with an A_{610}/A_{280} ratio of 0.040. It is noted that the coincidence is good except for peak IIa (fractions 285 305), where the A_{610}/A_{280} ratio is somewhat lower than 0.040. The discrepancy is evidently due to the presence of a small amount of colourless proteins with about the same chromatographic properties as ceruloplasmin IIa. This is shown by the ultracentrifugation analysis of material from the peaks in Fig. 21; two small components appear together with cerulolasmin IIa (Fig. 22). The

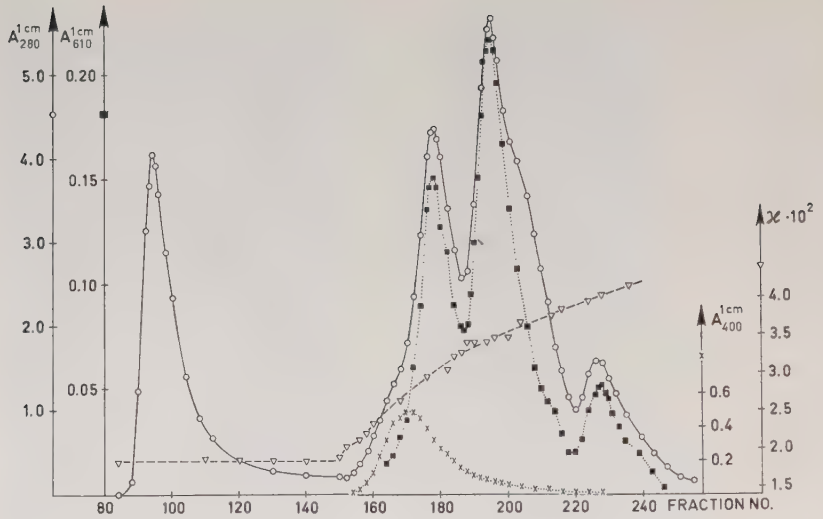

Fig. 23. Gradient elution on a 94×4.4 cm hydroxylapatite column of 2 g of Kabi DwZ 74. Gradient 0.125 →0.50 *M* (800+5320 ml.) potassium phosphate buffer, pH 6.8. Open circles: absorbancy at 280 mμ, filled squares: absorbancy at 610 mμ, crosses: absorbancy at 400 mμ, triangles: conductivity.

ultracentrifugation diagrams also show that the separation of the three peaks in Fig. 21 can not be due to polymerization (Table 2) which, however, may explain certain instances of ceruloplasmin heterogeneity reported in the literature (cf. below). Copper content and specific activity were approximately the same for the three ceruloplasmins (Table 2).

Table 2. *Comparison of ceruloplasmins I, IIa and IIb obtained by gradient chromatography on hydroxylapatite of purified ceruloplasmin preparations.*[a]

	I	IIa	IIb
Ratio[b] of copper content[c] to nitrogen content[d]	0.017	0.015	0.017
Ratio of activity[e] to copper content	4.0	4.8	4.1
Sedimentation constant[f], s_{20}	6.4	6.3	6.5

[a] See also Table 6.
[b] The ratio is expressed on a w/w basis.
[c] According to determinations with biquinoline (cf. ref. 27).
[d] According to Kjeldahl.
[e] Initial rates with standard solutions (see *Methods*, Chapter 4 and 1), except that EDTA was omitted (arbitrary units).
[f] Cf. Fig. 22 and ref. 14.

Care has to be exercised in making correlations between peak size ratios and functional states of the organism. Heavy loading of the columns, steep gradients and large fraction volumes will diminish the resolution of the chromatograms, and if ceruloplasmin IIa is present it tends to become assimilated with peak I, which makes the apparent I : II ratio too large, (I + IIa) : IIb being calculated instead of I : (IIa + IIb). On the other hand, the I : II ratio may be too small if the chromatography is preceded by unsuitable treatment of the material. This possibility is illustrated in Fig. 23 which shows the chromatogram of 2 g of the Kabi preparation DwZ 74. The latter was produced by re-purification involving ammonium sulphate precipitation according to (35) of stored older ceruloplasmin preparations (H. Björling, personal communication). It was noticed that the recovery according to copper determinations was unusually poor in this case. This observation and the unique shape of the A_{610} curve in Fig. 23 suggest that ceruloplasmin I was selectively destroyed during the preparation or during the intervening period of storage of DwZ 74 (Cf. also ref. 14). Observations on samples of ceruloplasmins I, IIa and IIb stored in phosphate buffers, pH 6.8 at about $+4°C$, repeatedly showed that the I variant is less stable than IIa and IIb. Ceruloplasmin I can gradually and irreversibly change into a yellow product with an absorbancy maximum at 412 mμ. This change is possibly preceded by a reversible reduction of the protein. The yellow protein is very tightly adsorbed to DEAE cellulose and can thus be separated from unchanged ceruloplasmin. Hydroxylapatite is less effective in this respect. DwZ 74 contained a certain amount of the yellow product which appeared at a slightly lower phosphate concentration than peak I (Fig. 23, crosses). If chloride ions are present in solutions of ceruloplasmin I, the transformation into the yellow species is delayd. Deutsch (59) also observed that ceruloplasmin is more stable in the presence of chloride than in phosphate at neutral pH.

Poulik (60—62) has investigated, by means of starch-gel electrophoresis, various forms of ceruloplasmin heterogeneity. According to him, several catalytically active forms with different mobility are probably polymers of ceruloplasmin. H. I. Gedin (pers. comm.) had the same experience with preparations in which alcohol precipitations at room temperature were used in order to remove contaminating proteins. This form of heterogeneity can be demonstrated by gel filtration (63).

Several of the artifacts described in the preceding sections are not likely to occur in analytical gradient chromatography of fresh serum. With regard to present knowledge of artifact-producing mechanisms, only a heterogeneity of ceruloplasmin obtained with fresh serum should be considered as due to a possible genetic multiplicity. In addition it would be necessary to use the conservative conditions observed in experiments of the type shown in Fig. 8. On the other hand, if a serum has been stored unfrozen for more than a few days, or if it has been frozen and thawed and if only two active components are obtained in the chromatogram (due to unsufficient resolution), allowance must be made for the uncertainty

of the degree of transformation of peak II material, and the uncertainty of the position in the chromatogram of the newly formed variant.

Ceruloplasmin in the normal newborn and in patiens with Wilson's disease

Works by Poulik (61, 62) indicate that ceruloplasmin can be dissociated into several non-identical structural subunits some of which are probably held together by weak forces. The genetic and pathological variants of this protein may be found at the level of its structural subunits. Thus far it is not known whether ceruloplasmin I and II represent different combinations of unequal polypeptide chains. A difference in the carbohydrate part could also account for the differing chromatographic behaviour. Thus, all of the 10 sialic acid residues can be removed without loss of catalytic activity (64).

Only newborns (see, for example, ref. 15) and patients with Wilson's disease (65) have serum ceruloplasmin concentrations definitely below the range of the normal adult. There is no simple explanation, such as nutritional deficiency or renal dysfunction, that can account for these circumstances. An attractive assumption is that the low values in these conditions are due to an inability to synthesize a component polypeptide chain. In the case of the newly born this is presumably because the corresponding gentic locus is still inactive awaiting signals to start producing a chain that exists only in "adult" ceruloplasmin. In the case of Wilson's disease the inability would be due to a permanent genetic deficiency. If this assumption is correct, one would expect a substantial deviation in sera of these individuals from the constantly reproducible shape of the ceruloplasmin pattern of normal adults (Fig. 8). Such was, however, not the case.

Fig. 24 shows the result of a hydroxylapatite chromatography with the gradient 0.065 →0.50 *M* of 5 pooled moderately hemolytic sera from cord blood. Corrections were made in the activity measurements for the absorbancy at 552 mμ caused by haemoglobin in the samples (cf. the cross curve, Fig. 24). The activity curve shows essentially the same configuration as normal adult curves.

Sera from 11 patients with Wilson's disease were analyzed in the hydroxylapatite gradient system. Several of these contained only traces of ceruloplasmin, and no conclusions could be drawn from the chromatographic picture although up to 55 ml of serum was used. In other cases sufficient amounts of the protein were present to allow recognition of an activity pattern. This did not appear significantly different from that of normal individuals. Examples are shown in Figs. 25 (patient V.C.) and 26 (patient M.P.) together with a chromatogram of an equally treated normal serum (J.G., Fig. 25). Patient V.C. had an unusually high

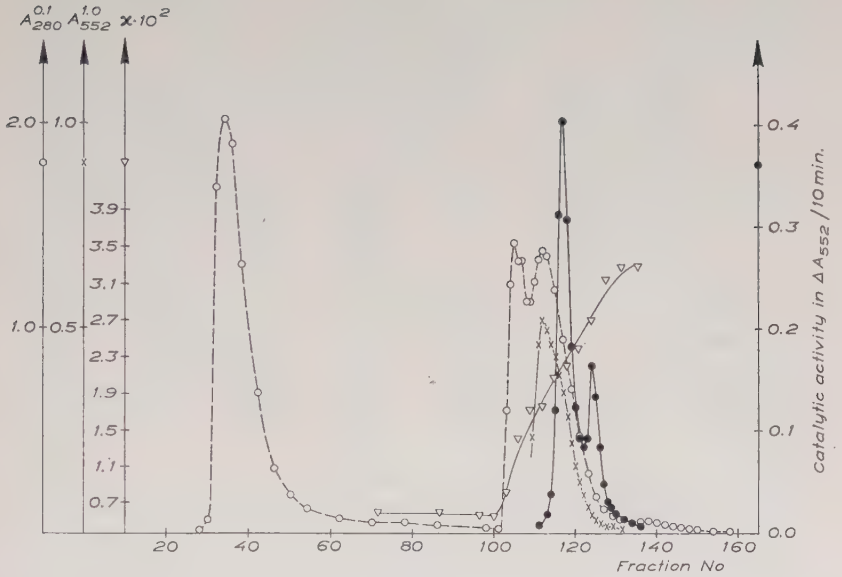

Fig. 24. Gradient chromatography on hydroxylapatite of 40 ml of fresh cord blood serum, pooled from 5 individuals. Column size: 28.5 × 1.6 cm. Gradient 0.065 → →0.50 *M* (78 + 283 ml) potassium phosphate buffer, pH 6.8. Open circles: absorbancy at 280 mμ, filled circles: activity (100 μl aliquots), crosses: absorbancy at 552 mμ, triangles: conductivity.

serum ceruloplasmin concentration (V.C. a) which increased further after estrogen therapy (V.C. b). For clinical data on these patients, see ref. 66. The reproducibility of the chromatographic method and of the ceruloplasmin assay method was checked with serum from patient M.P. (see legend of Fig. 26). Data on the experiments shown in Figs. 25 and 26 are collected in Table 3.

Hirschman *et al.* (67) obtained essentially the same result. They analyzed, by means of the hydroxylapatite gradient system, 10 pooled normal sera, 4 pooled sera from cord blood and one individual Wilson serum. Unlike the experiments described above, the material was submitted to a preliminary purification procedure involving frontal chromatography on DEAE-cellulose and on hydroxylapatite. Two ceruloplasmin-containing peaks appeared under the gradient, and the ratio of the concentrations of these two ceruloplasmins was found to be approximately 3 : 1 in all 3 cases. Richterich *et al.* (21) found that in normal adults the amount of ceruloplasmin in peak I was 31 ± 6 mg/100 ml of plasma and that the amount in peak II was 8 ± 2 mg/100 ml of plasma. The means for three pregnancy sera (15) were: peak I 63 mg/100 ml and peak II 23 mg/100 ml. On

Table 3. *Data on sera and serum chromatograms from one normal individual and two patients with Wilson's disease. Cf. Figs. 25 and 26.* (L. Broman and A. G. Bearn, unpublished work).

Subject	Serum		Gradient chromatography on hydroxylapatite				
	Total Cu μg/100 ml	Ceruloplasmin mg/100 ml[a]	Curve in Fig.	Relative activity of starting material[b]	Per cent recovery of active material[c]	Per cent of recovered activity	
						Peak I	Peak II
J.G. (normal)			25 J.G.	0.029	75.8	86	14
V.C. 22.11.'58	108	20		0.012	82.0	81	19
V.C. 24.11.'58	116	18.5	25 V.C.a	0.016	69.4	83	17
V.C.[d] 1.12.'58	186	30.6	25 V.C.b	0.030	85.4	82	18
M.P.	112	7.6	26	0.006			

[a] Manometric determination.

[b] Ratio of $\Delta A_{552}^{1\text{ cm}}$/10 min. (100μl aliquots) to $A_{280}^{0.5\text{ cm}}$; see *Methods,* Chapter 1.

[c] Sum of specific activity times volume over all fractions in per cent of specific activity times volume of starting material; activity determinations according to *Methods,* Chapter 1.

[d] Treated with ethinyl estradiol.

the other hand, Richterich *et al.* (68) claim that peak I was smaller than peak II—or entirely lacking—in sera from newborns and from Wilson's disease. However, comparatively small amounts of individual sera were analyzed in these experiments, and the activity curves were evidently very flat. This circumstance may have rendered a correct identification of the two peaks difficult and made quantitative determinations rather uncertain.

In the investigation by Hirschman *et al.* (67) referred to above, comparisons were made between ceruloplasmins from various gradient chromatograms with respect to the mobilities in starch-gel electrophoresis. Minor differences were obtained between peaks I and II of the same serum as well as between normal peak II and respectively Wilson peak II and peak II from cord blood. A comprehensive study of various physical and chemical data of two ceruloplasmins obtained with gradient chromatography on hydroxylapatite of a purified preparation has been published by Richterich *et al.* (21). Several differences were found. These findings as well as those of Hirschman *et al.* (67) by starch-gel electrophoresis should be estimated in the light of the considerable lability that apparently is a characteristic of ceruloplasmin.

The provisional conclusion from data so far obtained is that no definite

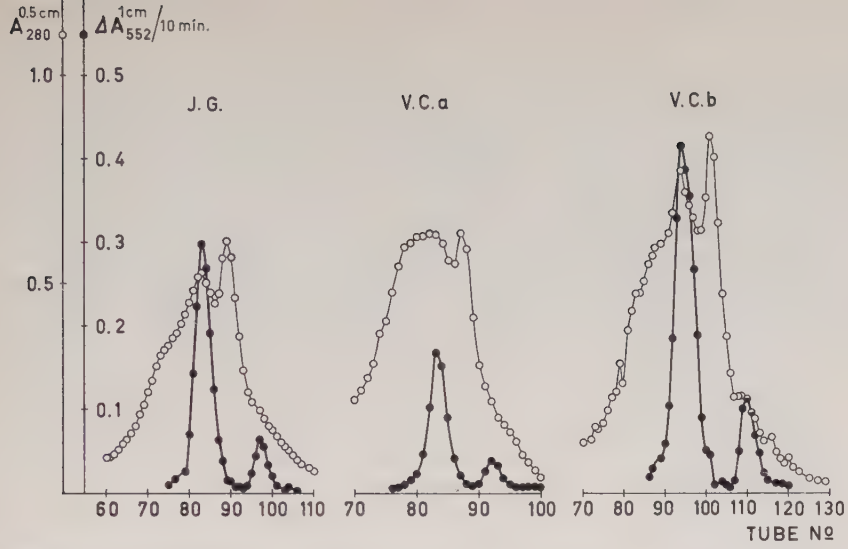

Fig. 25. Gradient elutions on hydroxylapatite of 5 ml of serum from respectively a normal individual (J. G.) and from a Wilson patient, before (V.C.a) and after (V.C.b) estrogen treatment (Cf. ref. 66). Gradient 0.125 → 0.50 *M* (78 + 283 ml) potassium phosphate buffer, pH 6.8. Open circles: absorbancy at 280 mμ. Activity: 100 μl aliquots for J.C. and V.C.b, 200 μl aliquots for V.C.a (filled circles). For comparison the activity values have been halved in the latter curve. Only the part of the chromatogram corresponding to fractions 40—70 in Fig. 8 is shown. Cf. Table 3. (L. Broman and A. G. Bearn, unpublished work).

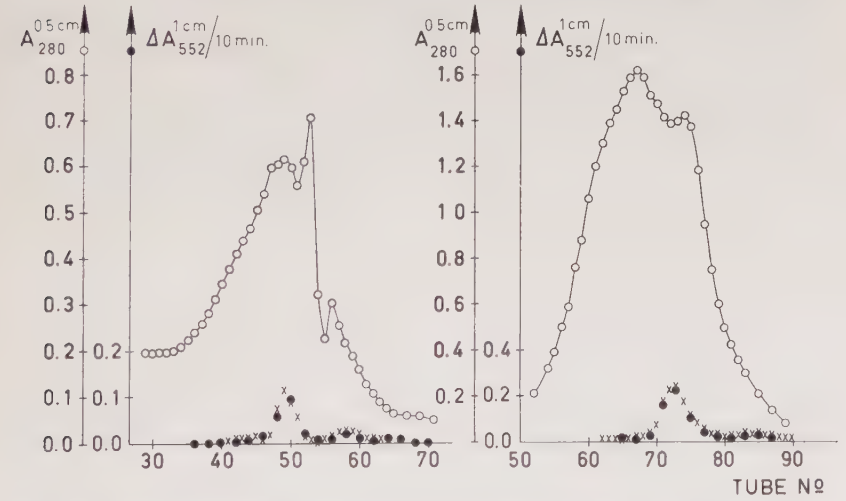

Fig. 26. Gradient elutions on hydroxylapatite of serum from a case of Wilson's disease (M.P., cf. ref. 66). Left curve shows the development of 5 ml of serum, right curve the development of 12 ml of the same serum. Gradient 0.125 → 0.50 *M* (78 + 283 ml) potassium phosphate buffer, pH 6.8. Activity (200 μl aliquots) was measured on two occasions (filled circles and crosses, respectively) in each case; the interval was 1 day for the left curve and 2 days for the right curve. Only the part of the chromatogram corresponding to fractions 40—70 in Fig. 8 is shown. Cf. Table 3. (L. Broman and A. G. Bearn, unpublished work.)

correlation has been proved to exist between the hereditary derangement in Wilson's disease and the heterogeneity of ceruloplasmin in the hydroxylapatite gradient system, as exemplified by the chromatogram in Fig. 8. The nature of the latter remains obscure. If peak IIb of purified ceruloplasmin is identical with peak II of fresh serum, then the possibility of a protein-protein interaction (41) is excluded according to the result of the ultracentrifugal analysis (Fig. 22). The heterogeneity might be due to the binding of a molecule or ion of low molecular weight to some of the ceruloplasmin (cf. ref. 8). However, the fact that the pattern is essentially the same in normal individuals and in Wilson cases does not necessarily exclude the possibility that the heterogeneity is of a genetic origin; the inherited metabolic error in Wilson's disease may pertain to some step in the (virtually unknown) mechanisms involved in the transfer of metabolic copper to the protein.

CHAPTER 3

CHROMATOGRAPHIC METHODS FOR THE PURIFICATION OF CERULOPLASMIN FROM HUMAN SERUM

Introduction

More than ten different methods have been devised for the purification of ceruloplasmin (for literature references, see ref. 35). The majority of these procedures depend on the use of various precipitation and denaturation stages, and some were intended for use on partly purified products, such as Cohn fraction IV-1.

The aim of the investigations summarized in the present chapter was to find an optimal combination of mild separation procedures that would allow large scale purification of ceruloplasmin directly from any given type of original human serum, for example, one representing a chosen pathological condition. Two purely chromatographic procedures were tried out (26, 30), each comprising only three steps. The methods were tested with retroplacental serum, which is particularly rich in ceruloplasmin (15, 35). No particular attention was payed to preexisting and induced heterogeneities in these experiments. The purification of peak II ceruloplasmin offers special difficulties, because of its lability (see Chapter 2). The purification method employed by Hirschman *et al.* (67, see Chapter 2) was in certain respects similar to those here described.

Method 1

As in the case of hydroxylapatite (37), ceruloplasmin is comparatively strongly adsorbed to DEAE ion exchangers (69). This makes the adsorbent indispensable in a set of chromatographic purification steps where a high capacity is desired. This tool has accordingly been employed in a number of purification schemes (*e.g.* 57, 59). The combination of DEAE chromatography and hydroxylapatite chromatography is advantageous not only for the reasons just mentioned, but also because the adsorption mechanisms are different in the two cases. As distinguished from DEAE resins, hydroxylapatite does not adsorb serum proteins with an order of preference that is regularly dependent on the positions of their isoelectric points (37). This fact increases the efficiency of removing contaminating proteins from ceruloplasmin when the two adsorbents are used in succession. For the

third stage of the first method the anion exchange resin Dowex-2, 200—300 mesh (Dow Chemical Company, Midland, Michigan) was employed. Ceruloplasmin is also comparatively strongly adsorbed to this resin (70) and it was expected that additional differences in the binding mechanisms would allow further separation of contaminating proteins. The pH was kept within the neutral range throughout, potassium phosphate buffer, pH 6.8, being used in all three steps. In one experiment the development in the last step was performed with Tris-(hydroxymethyl)aminomethane-hydrochloric acid (Tris-HCl) buffer, pH 7.2.

Hydroxylapatite was prepared as in Chapter 1 (p. 12) and DEAE-Sephadex as described in Chapter 2 (p. 29). The Dowex-2 pretreatment and column regeneration was with 1 *N* hydrochloric acid according to (70).

Plexiglass column tubes and a gradient mixing device of the types mentioned in Chapter 1 were used.

Analytical methods were the same as described in Chapter 1. Data on the degree of purification in various steps were based on the assumption that a 100% pure ceruloplasmin has an A_{610}/A_{280} ratio of 0.042 (30). It should be noted, however, that some authors present higher values for this ratio, thus Curzon and Vallet (57) claim 0.044, Blumberg *et al.* (72) 0.048, and Poulik (62) 0.047 or even 0.055. The reason for these discrepancies is not quite clear. One explanation could be that the ceruloplasmin under certain conditions is partly reduced. Furthermore, a series of experiments indicate that the absorbancy at 610 mμ is not quite proportional to the concentration of ceruloplasmin. The deviation from linearity is proportional to the concentration of ceruloplasmin and is dependent on the nature and concentration of the inorganic anions present, and on pH in the range 6.8 to 7.4. Thus, for example, dilution of a ceruloplasmin solution in 0.025 sodium phosphate buffer pH 6.8 with 10 times its volume of the same buffer will cause an exponentially time-dependent decrease of the absorbancy obtained immediately after the dilution. In one experiment the instantaneous absorbancy after dilution was $A^{1\,cm}_{610}=0.650$, and half the time-dependent decrement, approximately —0.025, was reached in about 3 minutes. The effect was even more conspicuous if the dilution was made with a phosphate buffer of pH 7.4. On the other hand, if the dilution was made with sodium chloride (about 0.02 *M* or more) no further decrease of the instantaneous value was observed. The mechanism is obscure but may be connected with variations of the degree of aggregation of the protein molecules. At any rate, estimates of ceruloplasmin concentrations based on the absorbancy at 610 mμ appear to be relatively unreliable. The final A_{610}/A_{280} ratio obtained by us in purification method 2 (below and ref. 30) never exceeded 0.042, although the preparation did not contain any contaminating proteins according to starch-gel electrophoresis and ultracentrifugation analysis.

Procedure and Results

Stage 1, the hydroxylapatite gradient chromatography, was described in Chapter 1 (Fig. 9, p. 20). About 90% of the total serum proteins passed through the column without being adsorbed and are not shown in Fig. 9. About 70% of the oxidase activity (filled circles) was recovered in the two peaks which coincide rather well with the blue colour (filled squares) of the ceruloplasmin. All fractions of peak I (tubes 238—260 in Fig. 9) were pooled (volume 275 ml) and dialyzed against 0.1 *M* potassium phosphate buffer, pH 6.8, for 27 hours with several changes of buffer.

Stage 2. 150 ml of the dialyzed solution was applied to a DEAE-Sephadex column with the dimensions 93×1.5 cm. The chromatogram was developed with the gradient $0.10 \rightarrow 0.30$ *M* potassium phosphate, pH 6.8 ($275 + 1100$ ml), and the result is given in Fig. 27. Four zones of protein appeared, and 70% of the blue colour (filled squares) of the ceruloplasmin applied was recovered in the last zone (fractions 142—180). The average degree of purification in this zone was about 50%.

Stage 3. Two alternatives were tested for the last stage. In one experiment pooled fractions 142—180 (volume 137 ml) of the chromatography shown in Fig. 27 was dialyzed for 20 hours against 0.05 *M* Tris-HCl buffer, pH, with several changes of buffer. The solution was applied to a column of Dowex-2 (75×3.3 cm) equilibrated with the same buffer. The

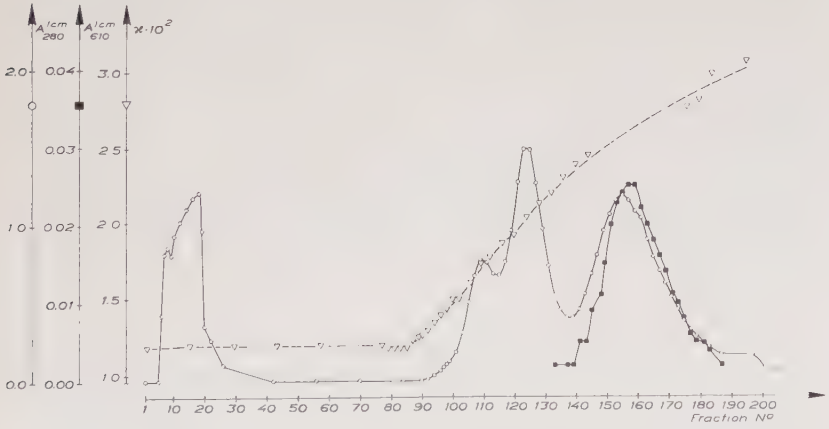

Fig. 27. Gradient chromatography on DEAE-Sephadex of material from ceruloplasmin peak I, the pooled fractions 238—274 from the experiment shown in Fig. 9. Column size: 92.5×1.5 cm. Gradient: $0.10 \rightarrow 0.30$ *M* potassium phosphate buffer, pH 6.8 ($275 + 1100$ ml). Open circles: absorbancy at 280 mμ, filled squares: absorbancy at 610 mμ, triangles: conductivity (26).

chromatogram was developed with stepwise increasing concentrations of Tris-HCl, pH 7.2. No protein appeared immediately after the void volume and only minute quantities after the application of a 0.22 *M* step. This was followed by a 0.26 *M* step in which both moderate amounts of contaminating proteins and some ceruloplasmin was eluted. Practically all of the ceruloplasmin was eluted by the following 0.33 *M* step. This corresponded to 71% of the applied amount of ceruloplasmin. The purity of the best fraction was 83%. In a second experiment about 35 mg of ceruloplasmin (calculated from $A_{610}^{1cm,\ 1\%} = 0.68$ (59)), with a purity of about 70%, was chromatographed on a 41.5×1.9 cm Dowex-2 column with the gradient $0.125 \rightarrow 0.50$ *M* potassium phosphate buffer, pH 6.8 ($275 + 1100$ ml). A blue zone appeared between the concentrations 0.19 *M* and 0.33 *M*, as calculated from the kappa-values (pH was, however, not checked). The degree of purification in the two best fractions was 95%, and one fully symmetrical peak only was obtained when one of these fractions was examined in the ultracentrifuge. These two fractions represented a recovery of 35% of the quantity applied in Stage 1.

Comment

It is obvious, for several reasons, that Method I does not satisfactorily meet the conditions made in the Introduction. In order to obtain a final purity of 80% or better, only a comparatively small number of the ceruloplasmin-containing fractions in steps 2 and 3 could be accepted without rechromatography (together with new starting material). This means that the overall capacity and the recovery was quite low for a single cycle. For example, to obtain the modest purity of 83% one was obliged to be content with the low overall recovery of about 35% per cycle. The capacity of the last step is rather limited, as is its contribution to the purification process. The use of the same buffer throughout, though rational in principle, is offset by the necessity of dialysis before steps 2 and 3 because of the varying buffer concentration in the gradient eluates. Some minor advantages might be gained by trying out optimal conditions for stepwise elution in all 3 stages.

Method 2

The method of choice for large scale chromatographic purification procedures is a suitable combination of columns arranged for protein-protein displacement developments (36, 70). Here the starting material is fed into the column at a constant comparatively low buffer concentration. Often very sharply separated adsorption zones are obtained, especially if diluted protein solutions are used. This is shown schematically in Fig. 13 (p. 27). One possible way to perform this type of chromatography is to

chose the buffer concentration and the size of the column as follows. The available amount of tightly adsorbed proteins in the starting material should be sufficient to cause complete displacement from the column of the protein to be purified. In the first stage the column is fed with starting material until the front of the zone containing the protein of interest has reached the lower end of the column. All effluent up to this point is discarded. The feeding is then continued until the rear of the zone of interest has reached the lower end of the column. The collected effluent now contains the protein to be purified together with proteins that are less tightly adsorbed, only. In the second stage, this quantity is fed into a new column with the same adsorbent, and equilibrated with the same buffer. The size of the second column must be such that available amounts of the protein to be purified is sufficient to displace all other protetins from the column. The feeding is stopped when the front of the protein of interest has reached the lower end of the column, and the column is washed with pure buffer of the same concentration. The effluent up to this point is discarded. In the third stage, the protein is desorbed with a concentrated buffer which gives a highly concentrated solution of the protein. A requirement for this mode of operation is that the protein to be purified can be conveniently localized on the column and in the eluate. This is easily fulfilled in the case of ceruloplasmin on account of its blue colour and catalytic activity (which can be assayed with the semiquantitative spot test according to (73)). An experiment based on this principle is shown in Fig. 28. After filtration of retroplacental serum through a layer of Pevicon and dilution with an equal amount of 0.36 *M* potassium phosphate buffer, pH 6.8, 3760 ml of this starting material was fed into a 95×4.4 cm hydroxylapatite column which had been equilibrated with 0.18 *M* potassium phosphate

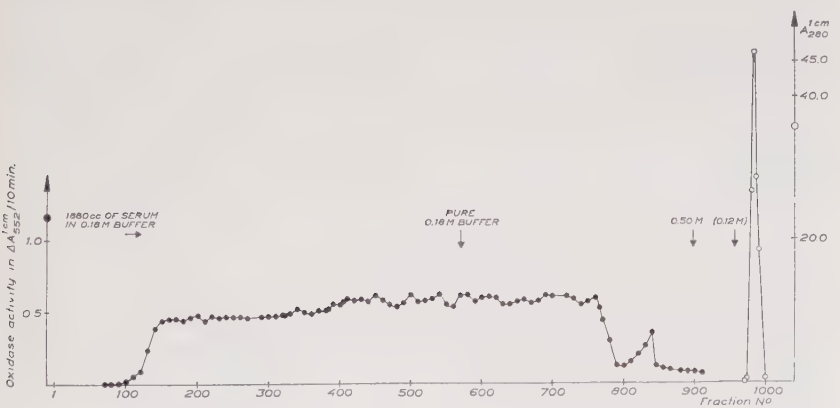

Fig. 28. Frontal analysis on hydroxylapatite of 1880 ml of retroplacental serum. Column size: 88×4.4 cm. Open circles: absorbancy at 280 mμ, filled circles: activity (100 μl aliquots).

buffer, pH 6.8. The eluate was taken up in a fraction collector, and every 10th tube was tested for ceruloplasmin activity (filled circles) with the standard method described in Chapter 1 (100 μl aliquots). The starting material was followed by pure 0.18 *M* buffer until the eluate no longer contained more than traces of activity. Adsorbed proteins (mostly β-lipoproteins) were then eluted by a 0.50 *M* step and were obtained as 380 ml of a highly concentrated, yellow, slightly opalescent solution (open circles). The fraction volume up to tube 700 was about 5 ml and after that successively increasing to about 10 ml. This explains partly the relatively small size of the second zone, fractions 800—850 (ceruloplasmin II. Cf. Chapter 1). A contributing circumstance was the fact that there was not enough serum available to effect complete displacement of adsorbed ceruloplasmin II from the column. The column was then equilibrated with 0.12 *M* buffer, and fractions 99—750 (3060 ml) were diluted with 1530 ml of distilled water to make the solution 0.12 *M* with respect to phosphate, after which it was again fed into the column. The adsorbed protein was washed with pure 0.12 *M* buffer and eluted with 1.0 *M* buffer, and a concentrated bluish green solution was obtained. The duration of the entire experiment was 47 days.

An attempt was made to use the β-lipoprotein-containing fractions 970—1000 (Fig. 28) as a displacer (36) in a separate experiment with a short hydroxylapatite column. This was, however, a failure because on rechromatography the β-lipoproteins showed changed chromatographic properties and were less tightly adsorbed than ceruloplasmin, presumably on account of the instability of β-lipoproteins (cf. 74). The observation is, however, of importance for the semicontinuous procedure (30) that emerged from these experiences. In this method ceruloplasmin contaminated with β-lipoproteins is returned to the container for starting material, which is thus possible without bringing about a corresponding accumulation of ceruloplasmin-displacing proteins.

The principle of *Method 2* is summarized in the schematic diagram in Fig. 29. Continuous operation is essential for obtaining a high recovery. Steps 1 and 3 consist of protein-protein displacement chromatographies, in both cases with ceruloplasmin as the developer which displaces less tightly adsorbed contaminants. DEAE-Sephadex columns are used in the first step and hydroxylapatite columns in the last step, in order to compensate, as far as possible, for the comparatively low flow rate in the latter. A selective desorption of ceruloplasmin is achieved in the first step by appropriate timing (for details see ref. 30). The highly concentrated effluent from the first step is utilized directly in the second step, which consists of gel filtration on Sephadex G-100 (63, 75). Various dilutions of potassium phosphate buffer, pH 6.8, are used throughout, with KCl added to stabilize ceruloplasmin (cf. Chapter 2). The entire process is performed at +4°C. Insufficiently purified ceruloplasmin fractions obtained at various stages are diluted to the appropriate ionic strength and re-utilized.

In a typical experiment the A_{610}/A_{280} ratio after the second step was

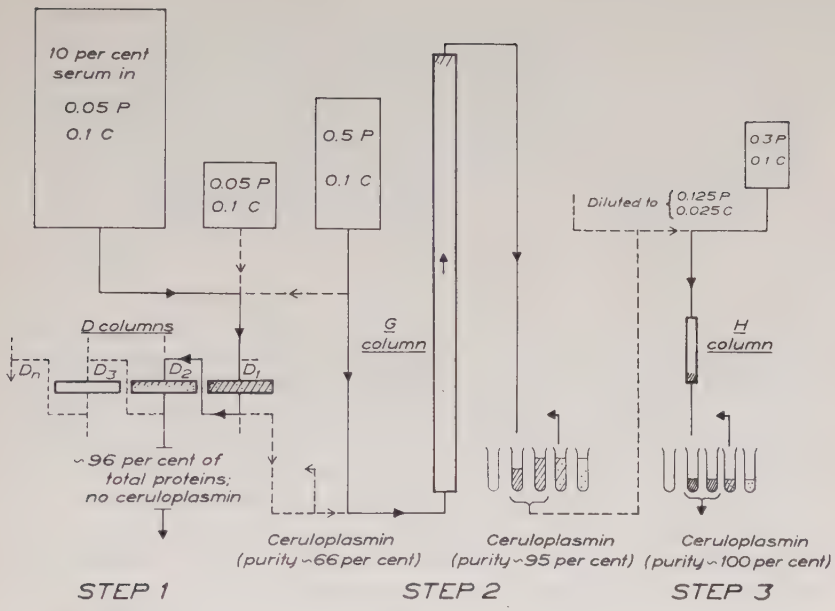

Fig. 29. Schematic diagram of the purification procedure of *Method 2*. D = DEAE-Sephadex, G = Sephadex G-100 gel. H = hydroxylapatite. P = potassium phosphate buffer (pH 6.8), C = potassium chloride. Numbers indicate molarity of solutions. Connections by full lines symbolize example of simultaneous operations in the three steps. *Step 1,* serum solution is passing through D_1 and D_2. D_1 is fully loaded with ceruloplasmin (oblique lines); some blue protein is also adsorbed in the upper part of D_2. *Step 2,* the gel filtration in column G is almost finished; 95% pure ceruloplasmin in fraction collector tubes, to the right (oblique lines), is ready for Step 3 after dilution; less pure material (oblique lines and dots) will be returned for re-utilization (return of material is indicated by right angle turned arrows). *Step 3,* elution of pure ceruloplasmin from a H column practically finished. Broken lines indicate connections at other stages of the process cycle. For example, in Step 1 the serum container is next connected to the inlet of D_2, the outlet of which is connected to the inlet of D_3. D_1 is washed with 0.05P—0.1C and then connected to the 0.5P—0.1C container. After partial desorption (see ref. 30) the first 2/3 of the eluate is fed into the lower end of the G column, and the rest is returned to the serum container (30).

0.040, which corresponds to a degree of purification of about 95%. After the third step the ratio increased to 0.042, corresponding to practically 100% pure ceruloplasmin. The overall recovery was about 85% when recycling of impure material was included. With a comparatively moderately dimensioned apparatus about 80 mg of pure material could be produced daily.

CHAPTER 4

THE RELATION BETWEEN BLUE COLOUR, CATALYTIC ACTIVITY AND MAGNETIC PROPERTIES OF CERULOPLASMIN

Introduction

It was mentioned in Chapter 1 that conspicuous changes of the absorbancy at characteristic wavelengths in the visible range of the spectrum provide a useful means of following oxidation-reduction reactions of ceruloplasmin and several of its substrates. As a matter of fact, the relatively small energy jumps corresponding to transitions in the visible and the near ultraviolet (34 to 114 kcal per mole) and the comparatively high intensity of the absorption, *i.e.* the high probability of such electron transitions, suggest a readiness for oxidation-reduction reactions. However, owing to difficulties of interpretation, optical spectroscopy is of limited usefulness in the study of the nature of oxidation-reduction mechanisms in complicated molecules such as ceruloplasmin. These problems are more directly approachable with certain methods that utilize the magnetic properties of such reactants.

A transfer of a single electron from one molecule to another is bound to cause a change of the magnetic properties of both of the reactants. The existence of an unpaired electron in an orbital is asssociated with paramagnetic behaviour of the species in question, *i.e.* a sample of it is attracted into the strongest part of an inhomogeneous magnetic field with a force that is proportional to the number of unpaired electrons in the sample and to the strength of the field times the field gradient. This principle is utilized in magnetic susceptibility determinations (see references in ref. 28) whereby the amount of unpaired electrons in a sample can be calculated, and information on oxidation states can be obtained. Thus, for example, the cupric ion contains one unpaired electron and the cuprous ion none; PPD and its *N*-methyl derivatives (see Chapter 1) have only paired electrons, whereas their first oxidation products each contain one unpaired electron.

The vast majority of electrons in organic substances are paired and comparatively unreactive. One could say that magnetic methods of investigation, as applied to the present problem, are selectively concerned with electrons that very probably are directly involved in relevant reactions of the compounds.

The relations of unpaired electrons to structures in which they move are advantageously investigated by means of another method that utilizes

the paramagnetic property, viz. electron spin resonance (ESR) spectroscopy (see, for example, introductory treatments in refs. 77 and 78 and reviews of biological applications in refs. 79 and 80).

The following brief survey of the principle is intended to provide a general background to the ESR spectra shown in Fig. 31.

Electron spin resonance spectroscopy

The method is based on the fact that the energy level of equivalent unpaired electrons is split in a homogeneous unidirectional magnetic field (which is provided by the ESR spectroscope) corresponding to the two possible orientations of their spin. An oscillating magnetic field, arranged perpendicularly to the D.C. magnetic field, will be attenuated if its energy equals the energy difference between the new levels, that is to say, there will be a net absorption of energy from this radiation in connection with transitions between the two spin orientations. The absorbance is registered by the instrument, analogously to the registration of absorption losses of monochromatic light passing a suitable sample in an optical spectroscope. Unlike orbital excitations in optical spectroscopy, the spin excitations in ESR spectroscopy occur between artificially produced energy levels. The spacing of the latter is dependent upon the strength of the unidirectional magnetic field. Thus, the resonance condition is fulfilled if

$$h\nu = g\beta B$$

where $h\nu$ is the energy of the oscillating field, β is a unit of magnetic moment and g is a measure of the interaction between the unidirectional field (of strength B) and the unpaired electrons.

The recording is performed, for example, by varying B continuously about a value of 3500 gauss while irradiating the specimen with a constant microwave frequency, $\nu = 10$ kMc/sec. The signal corresponding to the energy absorption at resonance is amplified and can be recorded directly *versus* B. But usually phase-sensitive detection is employed in order to improve the sensitivity of the method, in which case the first derivative of the absorption curve is obtained. The ESR spectra in Fig. 30e and 31 are of the derivative type.

The area under the absorption curve (or the doubly integrated derivative curve) is proportional to the amount of unpaired electrons in the sample, and absolute values can be obtained after appropriate calibration of the instrument.

The value of g in the above formula is 2.0023 for free electrons, and absorption bands located at D.C. magnetic field strengths (abscissa) that are associated with other g values indicate that the spin angular momentum of the electron is more or less strongly coupled with the orbital momentum. Experimentally determined g-values can give information on the immediate surroundings of a metal ion that has unpaired electrons, because

the contribution from the orbital motions of such electrons can be more or less suppressed by the electrostatic fields provided by the ligands.

An ESR parameter of particular interest in the present context is the hyperfine constant which refers to the coupling between the unpaired electron and the nucleus. Cu^{63} and Cu^{65} have a nuclear spin of 3/2, which means that the nuclei will orient their magnetic moments in either of 4 possible directions in relation to an outer magnetic field, such as the D.C. field of the ESR spectroscope. The fractions of otherwise equivalent nuclei in a sample that are oriented in each of these directions are nearly equal. The orientations of the nuclear moments are unaffected by the oscillating field. The projections of the nuclear magnetic moment in the direction of the applied field, corresponding to these orientations, are equally spaced. Two projections add to the strength of the outer field in the vicinity of the nucleus, and two subtract from it. Thus, there are four equally large poulations of cupric ions in the sample under analysis, each having a different local D.C. magnetic field in the vicinity of the nucleus. If the unpaired electron moves sufficiently close to the nucleus it will experience such a resultant field rather than the original D.C. field produced by the ESR spectroscope. This means that resonance will occur at four different values of the applied field, and the resultant ESR spectrum is actually the result of a superposition of four spectra which are separated in the direction of the abscissa. The separation of two successive component spectra is called the hyperfine constant and is expressed as a field strength (gauss) or a wave-number (cm^{-1}). If the spectrum is well resolved the separation is shown by a quadruplication of the absorption peak which is called the hyperfine structure (Cf. left part of the spectra in Fig. 31). The hyperfine constant can then be determined by measuring the distance between two vicinal hyperfine components (Fig. 30d and e). The longer the distance between the nucleus and the orbital containing the unpaired electron, the less will be the effect of the nuclear magnetic field, and the lower will be the value of the hyprefine constant. If the unpaired electron moves in an molecular orbital passing several nuclei with magnetic moments, there will be a corresponding increase of the number of hyperfine lines. The number of these lines is characteristic of the nuclei involved. It may thus be possible to deduce from the ESR data the degree of covalency of a bond, and the nature of the bonded atoms.

In the case of complexes with low symmetry, such as those of the cupric ion, both g-values and hyperfine constants show anisotropy; the former are denoted $g_{\parallel}$ and $g_{\perp}$, and the latter A and B (see Fig. 30). Owing to insufficient resolution, it was not possible to measure B in the experiments to be described in this chapter.

ESR studies on ceruloplasmin

Originally ceruloplasmin was characterized as a laccase (6) on the basis of similarities in substrate specificity with the plant enzyme of that name. Laccase is a blue protein which contains 4 atoms of copper per molecule. Sub-

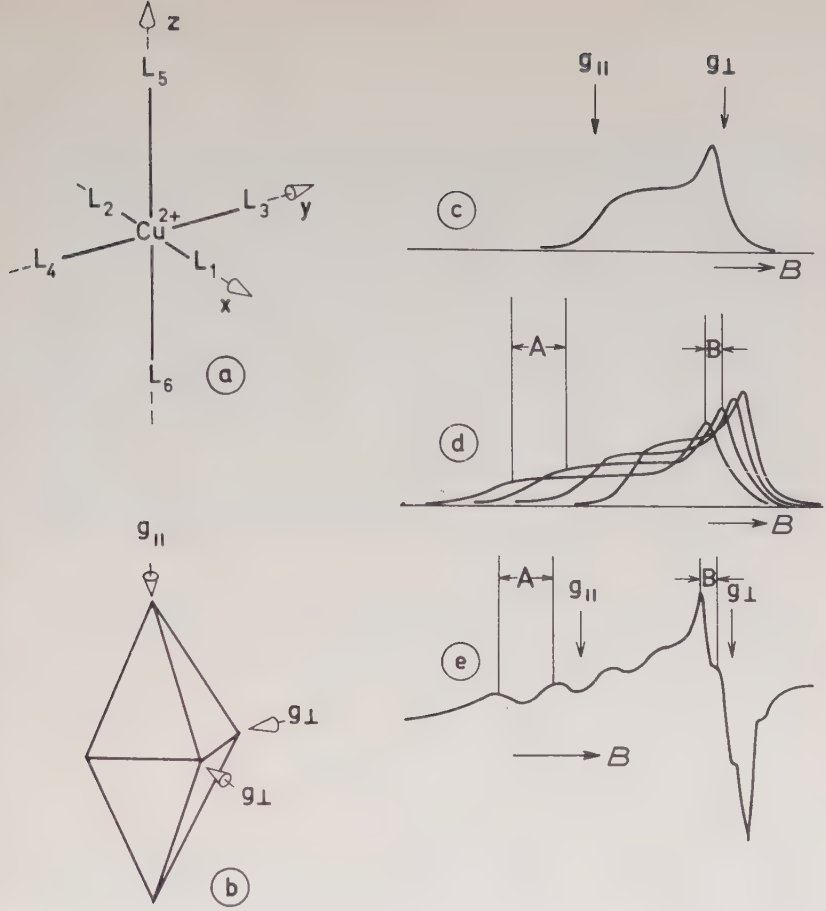

Fig. 30. Schematic diagrams to illustrate the generation of the ESR spectrum of a cupric complex.

a) Tetragonal symmetry found in most Cu^{2+} complexes. Ligands L_1—L_4 comparatively close to the central ion. The 3d orbital with lobes along the x and y axes is strongly influenced by the electrostatic field of these ligands. This orbital has the highest energy and contains the unpaired electron.

b) Distorted octahedron corresponding to the symmetry shown in (a). Measurements of **g** on a single crystal containing such units (diluted with an isomorphous diamagnetic compound) will give different values depending on the direction of the D.C. magnetic field in relation to the axis of symmetry ($g_{\parallel}$ = parallel to the axis, $g_{\perp}$ = perpendicular to the axis). The positions of the anisotropic **g**-values are indicated by arrows in (c). Note that $g_{\perp}$ is isotropic in the xy plane (b).

c) Absorption band obtained from a powder of randomly oriented crystals (hyperfine structure disregarded). The absorption intensity corresponding to predominantly perpendicular orientation of the axis of symmetry to the applied field is comparatively high due to the isotropy of $g_{\perp}$ in the xy plane. Similar spectra are obtained from solutions of macromolecules containing the paramagnetic ion, because the rotation of such molecules is too slow to average out the anisotropy (see ref. 81).

d) The hyperfine structure is due to a quadruplication of the absorption band corresponding to the space quantisation of the nuclear momentum of copper (see text).

e) First derivative of the four superimposed curves indicated in (d).

sequent investigations by Malmström and Vänngård (81) have revealed additional common traits in these two copper proteins. The authors studied, by means of ESR, a number of small-molecule copper complexes and some copper proteins (laccase, ceruloplasmin, erythrocuprein and Cu^{2+}-carboxypeptidase). It was shown that the native oxidized forms of ceruloplasmin and laccase contain bivalent copper, and that this ion changes valency when the proteins are transformed to their leuco forms in the presence of a substrate. Compared to all other substances investigated, ceruloplasmin and laccase showed a strikingly narrow hyperfine structure. The A-values (cf. Fig. 30) for these proteins were about 0.009 cm^{-1}, whereas all other substances gave A-values between 0.015 and 0.022 cm^{-1}. On denaturation, ceruloplasmin and laccase gave the broad hyperfine structure typical of the other complexes (cf. Fig. 31). These findings were tentatively interpreted as due to a high degree of delocalization of the unpaired hole on Cu^{2+} of native ceruloplasmin and laccase. This interpretation agrees well with the catalytic properties of these proteins.

There may thus be a connection between the catalytic activity of ceruloplasmin, its intense absorption in the visible region and its unique ESR hyperfine structure. The present investigation (27) was undertaken in order to further elucidate this problem.

Materials and methods

Proteins and reagents

Kabi ceruloplasmin, prepared from human retroplacental serum according to Björling (35), was further purified and separated into three active peaks by gradient chromatography on hydroxylapatite, as described in Chapter 2 (Fig. 21 p. 36). In most experiments the best fractions from peak I were used, but in some experiments a mixture of all three forms of ceruloplasmin was employed, after removal of inactive material by stepwise hydroxylapatite chromatography. The A_{610}/A_{280} ratio of the material was > 0.040. Chromatographic fractions were concentrated according to (76) or (82) to a final protein concentration of about 8×10^{-4} *M*, as determined by copper analysis on the basis of a copper content of 0.34% and a molecular weight of 151.000 (8, cf., however, 14). The concentrated solution was dialysed for about 24 hr. at 4°C against 10 times its volume of sodium phosphate buffer, pH 6.8, sometimes including 0.01 *M* NaCl to stabilize the protein (see Chapter 2). In some cases EDTA was also included in the buffer in order to remove extraneous copper. The concentrations of buffer and EDTA varied in separate experiments, and details are given under *Results*.

De-ionized water was used in making up all solutions. Buffer solutions were purified by dithizone extraction (89). Reagent-grade urea was recrystallized in water before use.

Activity measurements, visible spectra and optical rotation

The activity was measured spectrophotometrically at 25°C with *N,N*-dimethyl-*p*-phenylene diamine as substrate and calculated as the change in the absorbancy per minute at 323 mμ in a 1 cm cell. In general, the absorbancy increase was linear with time during the period of measurement. A Beckman DU-spectrophotometer, provided with thermospacers and coupled to a potentiometric recorder, was employed, and 25 or 50 μl of enzyme solution was added to 3 ml of substrate mixture with a plastic plunger. The composition of the substrate mixture was the same as described in Chapter 1. In the experiments with urea denaturation, no urea was included in the substrate since no regain of activity on dilution could be observed.

Visible spectra and ultraviolet difference spectra were recorded at 22°C in a Zeiss RRQ2OA recording spectrophotometer. This photometer, with the drum connected to a slow constant-speed motor, was also used for con-

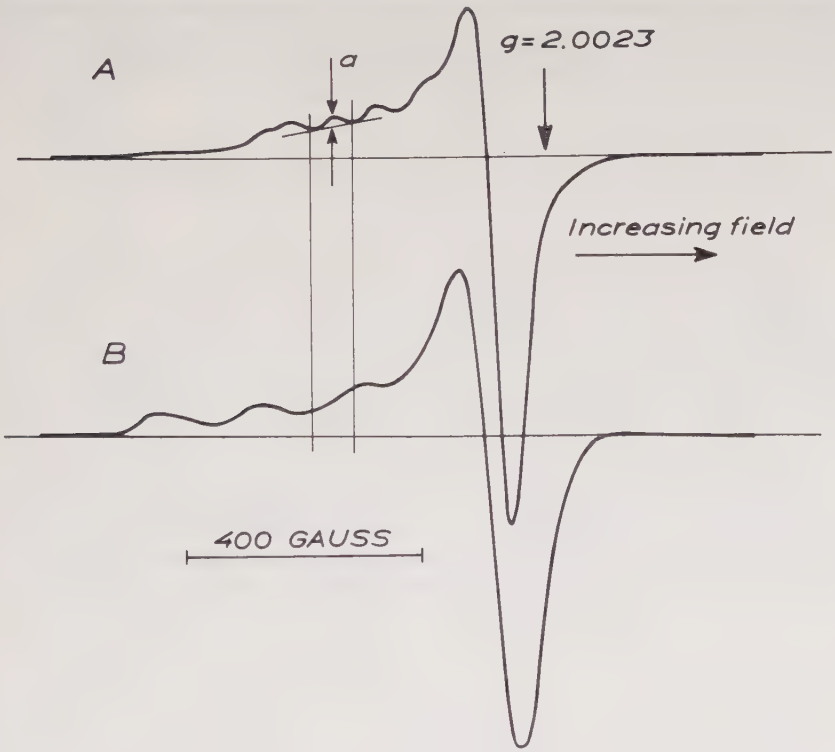

Fig. 31. ESR spectra of frozen solutions of native *(A)* and urea denatured *(B)* ceruloplasmin (conc. 5×10^{-5} *M*) in 0.025 *M* phosphate buffer, pH 6.8; the solution of denatured protein contains 7.3 *M* urea. The distance *a* was used as a measure of the intensity of signal A in the denaturation experiments (27).

tinuous recording of the absorption at 610 mμ in denaturation experiments.

The optical rotation at 326 mμ was measured in a recording Rudolph spectropolarimeter with a 5 cm cell. The performance of the optical rotation determinations by Dr. A. Rosenberg is gratefully acknowledged.

ESR measurements

The ESR spectra shown in Fig. 31 were obtained at liquid nitrogen temperature from native (curve *A*) and fully denatured (curve *B*) ceruloplasmin, respectively. However, intermediate degrees of denaturation gave in no instance a spectrum with an intermediate degree of hyperfine splitting. Recordings from samples at various stages of denaturation were therefore compounded of various contributions from *A* and *B* spectra. In measuring the intensity of spectrum *A* in the presence of the overlapping spectrum *B*, advantage was taken of the fact that a characteristic detail of spectrum *A*, between the vertical lines in Fig. 31, appeared in a region of the magnetic field where the derivative of spectrum *B* is approximately a linear function of the magnetic field. Thus, the distance *a* in Fig. 31, gives a measure of the intensity of signal *A*. (For further data on the ESR technique used, see ref. 27).

Effect of gradual urea denaturation on several characteristics of ceruloplasmin

Various stages of ceruloplasmin denaturation were achieved 1) by exposing the protein to a relatively low urea concentration for different lengths of time, and 2) by allowing various urea concentrations to act on the protein during a constant interval. The following parameters were determined: 1) absorbancy at 610 mμ, 2) absorbancy at 287 mμ, 3) optical rotation at 326 mμ, 4) catalytic activity, and 5) intensity of the ESR signal characteristic of native ceruloplasmin (Fig. 31, spectrum *A*).

The time course of the changes of parameters 1, 4 and 5 in the presence of 3.6 *M* urea is illustrated in Fig. 32. The rates of change under exposure to this concentration of the denaturant are sufficiently slow for convenient performance of the operations involved. The protein used in this experiment had been dialyzed against 0.025 *M* phosphate buffer, pH 6.8, containing 0.001 *M* EDTA. The absorption at 610 mμ was followed continuously at 26°C, and at given intervals samples were removed for activity and ESR measurements. The denaturation reaction was quenched by freezing these samples in liquid nitrogen. Two 50-μl aliquots were used for the activity measurements, and these were assayed within 1 minute after thawing; the freezing and thawing did not appreciably effect the activity. The samples for the ESR measurements were pipetted directly into the ESR sample tubes and kept in liquid nitrogen until analyzed in the ESR spectrometer.

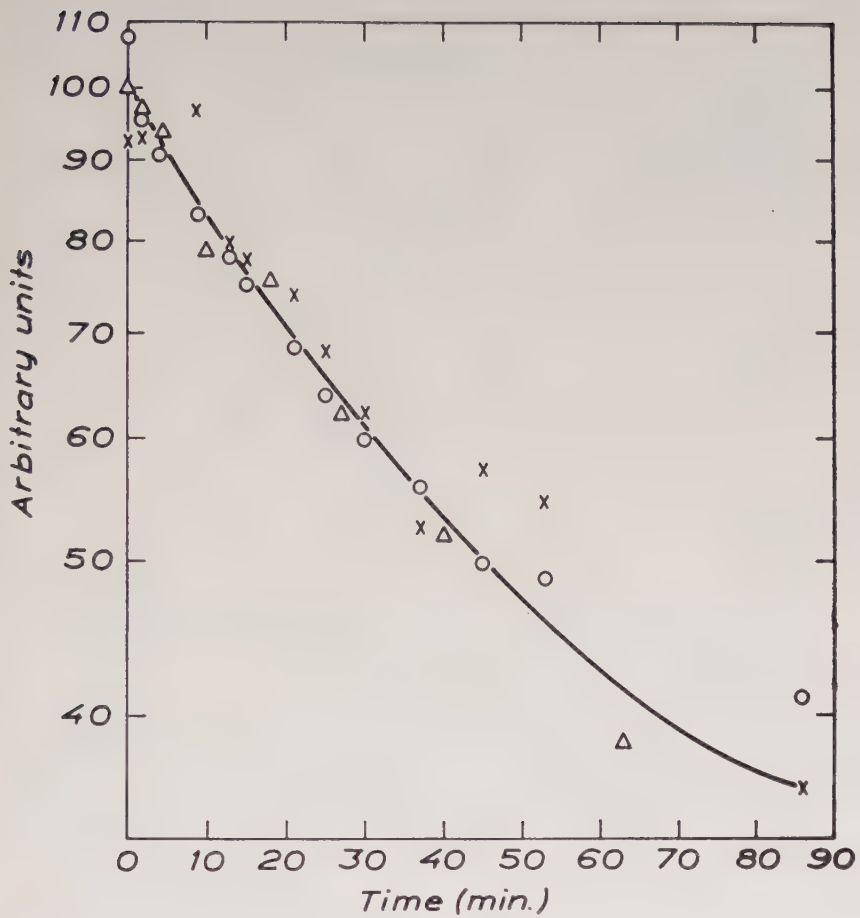

Fig. 32. The effect of 3.6 *M* urea at 26°C on the absorbancy at 610 mμ (circles), the distance *a* (triangles, see Fig. 31), and the activity (crosses) of ceruloplasmin (conc. 5×10^{-5} *M*) in 0.025 *M* phosphate buffer, pH 6.8, containing 0.001 *M* EDTA. The experimental values of all three parameters are expressed in arbitrary units and plotted on a logarithmic scale (27).

Fig. 33 shows the changes induced in the same three parameters by various concentrations of urea acting on the protein during a constant time interval. The protein used in this experiment had been dialyzed against 0.1 *M* phosphate buffer, pH 6.8, containing 0.01 *M* NaCl. The ceruloplasmin solutions with varying urea concentrations were allowed to stand for 24 hours at 22°C before the measurements were made, since no

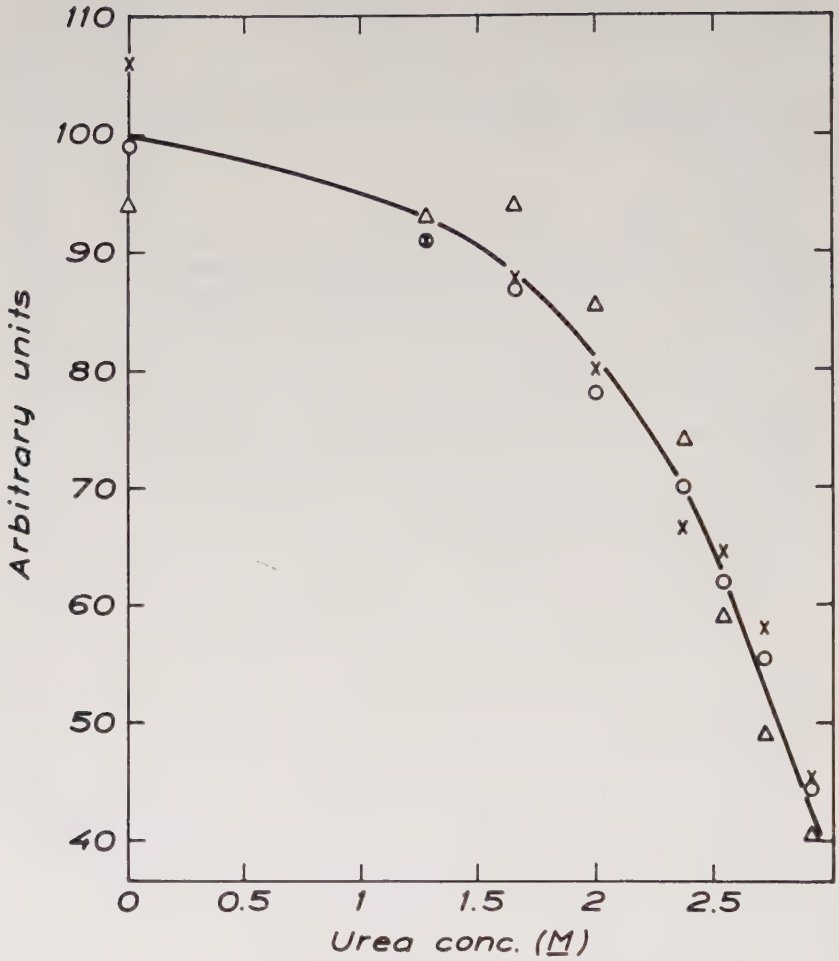

Fig. 33. The effect of urea concentration after 24 hr at 22°C on the absorbancy at 610 mμ (circles), the distance *a* (triangles, see Fig. 31), and the activity (crosses) of ceruloplasmin (conc. 5×10^{-5} *M*) in 0.10 *M* phosphate buffer, pH 6.8, containing 0.01 *M* NaCl. The experimental values of all three parameters are expressed in arbitrary units (27).

appreciable further change in visible absorption occurred with longer times. The spectrum of each solution was recorded in the wavelength region 500 to 800 mμ with the same concentration of protetin in 7.3 *M* urea as blank, after which equal portions were pipetted into the ESR sample tubes and frozen in liquid nitrogen. The activities of all solutions were determined within approximately 1 hour using 25 and 50 μl samples.

Table 4. *The effect of urea at 27°C on the optical rotation at 326 mμ (α) and absorbancy at 610 mμ (A_{610}) of 6.6×10^{-5} M ceruloplasmin in 0.025 M phosphate buffer, pH 6.8* (27).

Urea concn. (*M*)	Time (min)	$-\alpha$ (degrees)	A_{610} (1 cm cell)	Change in % of maximum change (7.3 *M* urea)	
				α	A_{610}
3.1	0	0.260	0.68	—	—
3.1	2	0.260	0.65	0	4
3.1	29	0.274	0.50	9	26
3.1	49	0.280	0.45	13	34
3.1	72	0.284	0.42	16	38
3.1	152	0.292	0.36	21	47
3.1	182	0.294	0.35	23	49
3.64	0	0.260	0.680	—	—
3.64	2	0.280	0.538	13	21
3.64	7	0.286	0.470	17	31
3.64	12	0.292	0.422	21	38
3.64	185	0.340	0.190	53	72

Table 4 compares the changes with time of parameters 1 and 3 at two different urea concentrations, and the results are also expressed in per cent of the maximum change obtained with 7.3 *M* urea. The optical rotation $\left[\alpha\right]^{27}_{326}$, was —52° without urea and —82° in 7.3 *M* urea. It should be noted that corrections have not been made for differences in refractive power of the solutions. The protein concentration and other conditions were comparable with those in the experiment depicted in Fig. 32, the main difference being the higher temperature and the exclusion of NaCl.

Table 5. *The effect of urea concentration after 24 hr at 22°C on the absorbancy at 287 mμ of ceruloplasmin (concn. 5×10^{-5} M) in 0.10 M phosphate buffer, pH 6.8, containing 0.01 M NaCl* (27).

Urea concn. *M*	Absorbancy difference, native *vs.* urea-treated protein (0.2 cm light-path)	Change in per cent of maximum change (7.3 *M* urea)	
		Absorbancy difference	Parameters in Fig. 38
2.5	< 0.002	< 1.6	36[a]
3.0	0.002	1.6	62[a]
5.0	0.074	60	> 93[b]
7.3	0.123	100	100

[a] Read from the curve in Fig. 33.
[b] This value was obtained with 4.36 *M* urea.

Table 5 compares parameter 2 with parameters 1, 4 and 5 (as read from the curve in Fig. 33) with various urea concentrations acting on the protein for 24 hours. Difference spectra in the region 250 to 310 mμ of native ceruloplasmin against urea-treated ceruloplasmin showed two main peaks at 287 and 292 mμ, respectively, the former being the largest. No detectable change occurred below a urea concentration of 2 *M*. The absorbancy differences at 287 mμ with some higher urea concentrations are shown in the table, and the changes in per cent of maximum change (with 7.3 *M* urea) are given. Experimental conditions were the same as those pertaining to Fig. 33. Due to the high protein concentration a 0.2 cm light path was used; with this a slit width of less than 0.4 mm was obtained in the whole region 250 to 310 mμ when the spectrophotometer was operated at high sensitivity.

Discussion

According to current theory, protein denaturation by urea is a complex process in which the conformation of the molecule is progressively destroyed through a succession of small changes (see, for example, ref. 83). This conception is supported by the fact that the changes of different properties of a protein undergoing urea denaturation at a measurable rate can show different concentration dependency and time course. Thus, urea often inactivates enzymes at concentrations which cause little change in optical rotation (see, for example, ref. 84). Conversely, a consistently parallel change of several parameters during gradual urea denaturations performed in different ways suggest that all of these parameters represent the same conformal quality. The experiments illustrated in Figs. 32 and 33 show that, within the limits of error, the same degrees of de-colourization, of activity loss and of transition from the native to the denatured type of ESR spectrum are obtained over a wide range of denaturation stages which were produced by varying the denaturation time and the concentration of the denaturant. On the other hand, results collected in Tables 4 and 5 show that two other parameters, optical rotation and absorbancy at chosen wavelengths in the near ultraviolet region, were otherwise dependent on the stage of denaturation. These results make it appear very likely that the strong visible absorption of ceruloplasmin is related to the uniquely bonded Cu^{2+} ion (81), and, furthermore, that the unique bonding is necessary for catalytic activity.

As was mentioned under *ESR measurements,* the transition from the native type of ESR spectrum (*A* in Fig. 31) to the denatured type (*B* in Fig. 31) occurs without formation of measurable quantities of species displaying intermediate degrees of hyperfine splitting. It would thus appear that there is an abrupt transition in the bonding of Cu^{2+} from the state giving spectrum *A* to that giving spectrum *B*.

C. B. Kasper *et al.* (85) have recently described similar changes of the

ESR spectrum of ceruloplasmin in connection with a number of treatments that change the charge, shape, colour and activity of this protein (Cf. Chapter 2).

It is of some interest in the present context that Curzon (86) obtained a partially reversible de-colourization, loss of activity and $A \rightarrow B$ transformation of the ESR spectrum of ceruloplasmin by lowering the pH from 7.0 to 3.5 and restituting it within various short time intervals (order of minutes). In addition, it was observed that the residual colour changed reversibly towards green at pH 3.5. It was mentioned in Chapter 2 that ceruloplasmin I can be transformed into a yellow variant if stored at neutral pH in phosphate buffer. Transformation of ceruloplasmin into a yellow form was frequently observed in connection with the urea denaturation experiments described in the present chapter (cf. also ref. 8). The nature and the interrelations of these observations are obscure.

The experiments described in this chapter indicate that the typical blue colour of ceruloplasmin is associated with the cupric ion. A great many complexes and compounds containing divalent copper are coloured in some shade of blue, corresponding to an absorption in the 600—900 mμ region of the spectrum. Absorption in the visible region of the spectrum by the cupric ion is often due to transitions of electrons within the 3d subshell, whose orbitals differ slightly in energy due to effects of the electrostatic fields of the ligands (see, for example ref. 87 and Fig. 30a). These d-d transitions are however, essentially "forbidden" by the rules of quantum mechanics, and the corresponding molar extinctions are accordingly low (the order of 100). The extinction coefficient of ceruloplasmin is, on the other hand, of the order of 1000 (72). Such large coefficients of a cupric complex (presumably lacking other types of relevant chromophoric groups) may indicate the existence of a so-called charge-transfer mechanism (87). This is, in principle, an electronic transition between two orbitals, such that the orbital of the excited state is localized on one atom or group (the acceptor) and the orbital with lower energy is localized on a different atom or group (the donor). The connection with oxidation-reduction mechanisms is obvious. Of particular interest for the present problem is the fact that the cuprous ion can act as a charge donor in a charge-transfer complex. This is, for example, probably the case with oxygenated haemocyanin (88), where the loosely bound oxygen molecule is the acceptor. The question of whether ceruloplasmin contains cuprous charge donors forms the topic of the next chapter.

CHAPTER 5

THE VALENCY OF COPPER IN THE OXIDIZED FORMS OF CERULOPLASMIN AND LACCASE

Introduction

While ESR studies on the oxidized forms of native ceruloplasmin and laccase (27, 81, 90) show unequivocally that these molecules contain at least one unpaired electron associated with a copper nucleus, and thus are cupric complexes, the experiments so far described were not concerned with the actual number of cupric and cuprous ions per molecule of the oxidized forms. Several strictly chemical approaches to valency determinations on metalloproteins are given in the literature, for example, such as that employing the specific Cu^+ reagent biquinoline (91). However, the use of valence-specific reagents in the analysis of metalloproteins is liable to give ambiguous results, for example, through oxidizing or reducing groups present, such as -SH (cf. refs. 92 and 93).

C.-B. Laurell (8) employed the indirect method of optical spectroscopy on ceruloplasmin at various stages of reduction. He found that titration of oxygen-free solutions of the protein with reducing solutions was accompanied by a proportional decrease of absorbancy at 610 $m\mu$ and a consumption of the reducing substance. The minimum amount of electron donor necessary to cause complete disappearance of the absorbancy at 610 $m\mu$ was found to be equivalent with the amount necessary to reduce four cupric ions per ceruloplasmin molecule to the cuprous state.

For the present problem ESR and magnetic susceptibility measurements are superior to the above-mentioned procedures, due to the fact that they provide direct information on the state of the metal ion without any interference with the reactions of the native protein. The results obtained by us with these techniques (27, 28) are in accordance with those of Laurell (8) mentioned above, which indicates that only half the number of copper ions in the oxidized form of ceruloplasmin are bivalent. This proportion was also obtained in experiments with fungal laccase. Current interpretations of relations between the mixed copper valency in these proteins and, on the other hand, the intense visible absorption and the unique hyperfine structure (see Chapter 4) will be discussed. Further aspects on this question are provided by a comparison with the characteristics of azurin (29, 94), a blue copper protein of bacterial origin which contains only one copper ion per molecule.

Materials and Methods

Proteins

Ceruloplasmin was prepared as described in the preceding chapter. In some experiments material from the three active peaks in the hydroxylapatite chromatogram (cf. Fig. 21, page 36) was tested separately. Apoceruloplasmin was prepared by dialyzing 3 ml of ceruloplasmin I (about 6% protein in 0.025 *M* sodium phosphate buffer, pH 6.8) at 4°C for 3 days against 3 changes of 500 ml of 1 *N* KCN in 0.05 *M* tris(hydroxymethyl)aminomethane-HCl buffer, pH 8.0, followed by dialysis against 4 changes of 0.025 *M* sodium phosphate buffer, pH 6.8, for an additional 3 days. No copper was detected in the dialyzed protein which, with the amounts analyzed, means that the copper content was $< 0.5\%$ of that of native ceruloplasmin. Laccase *A* and *B* from *Polyporus versicolor* was prepared according to (95) and concentrated as described for ceruloplasmin in Chapter 4. The purified enzymes gave single symmetrical peaks in the ultracentrifuge. We are indebted to Dr. R. Mosbach for his assistance in the purification work.

Copper analyses

Analysis of copper was carried out through determination of Cu^{1+} with 2,2′-biquinoline after reduction of bivalent copper, or, occasionally, by the dithizone method after wet digestion. For details, see ref. 27.

In regard to the removal of paramagnetic contaminants from reagents see Chapter 4. Reagent grade copper salts and perchloric acid were used without further purification.

ESR and magnetic susceptibility measurements

ESR spectra were recorded at liquid nitrogen temperature, and the absorption intensity was calculated from the derivative curve as described in ref. 27. Cupric complexes with histidine or EDTA were used as standards

Magnetic susceptibilities were determined at 20°C according to (96) and (97). See ref. 28 and experimental details under *Results*.

Results

Table 6 shows ESR data on the concentrations of cupric ion in respectively ceruloplasmin and laccase, in the native state and after treatment with perchloric acid. In the former case, both protein samples were pretreated by dialysis against 0.10 *M* phosphate buffer, pH 6.8. In addition, the ceruloplasmin solution had previously been dialyzed against this buffer with 0.005 *M* EDTA added in order to remove any contaminating copper. The perchloric acid-treated samples were produced from these same solu-

Table 6. *Concentrations (m-molar) of copper in native and perchloric acid-treated ceruloplasmin and laccase, corresponding to the observed ESR signals and obtained by chemical analysis* (27).

Experimental parameter	Ceruloplasmin				Laccase	
	Kabi	I	IIa	IIb	*A*	*B*
ESR signal of native protein	0.99	2.96	1.84	0.77	0.90	0.46
ESR signal of perchloric acid-treated protein[a]	1.74	5.39	—	1.37	2.10	0.87
Total Cu (chemical analysis)	1.92	6.60	3.86	1.68	2.10	1.08
Ratios						
native/acid-treated	0.57	0.55	—	0.56	0.43	0.53
native/total	0.52	0.45	0.48	0.46	0.43	0.43

[a] Values corrected for dilution of sample.

tions by mixing the samples with equal volumes of 70 to 72% perchloric acid and warming on a water bath to about 35°C. The clear, slightly yellowish-green solution was transferred to the ESR quartz tubes before cooling which caused a reversible precipitation to appear.

Weak ESR signals of type *B* (Fig. 31) were often obtained from native oxidized or reduced forms of ceruloplasmin and laccase. Their intensity corresponded at the most to 10% of the total copper present, and corrections for this absorption have not been made in Table 6. The results of chemical analysis of total copper in the samples are included in the table.

Table 7. *Susceptibility data for ceruloplasmin and laccase at 20°C* (28).

Sample	Protein concn. (mg/ml)	Copper concn. (m$_M$)	Susceptibility difference measured	$\chi_M \cdot 10^6$ c.g.s. e.m.u.
Laccase *A* (prep. 1)	52.1	3.46[a]	χCu,ox—χCu,red	680(±100)
Laccase *A* (prep. 2)	50.0	3.70	χCu,ox—χCu,red	570(±60)
			χCu,ox—χCu-free	550(±20)
Ceruloplasmin I	67.7	3.50	χCu,ox—χCu,red	430(±80)[b]
			χCu,red—χCu-free	120(±60)

The errors given are estimated maximal deviations.

[a] This value is probably about 10% too low since it was obtained by the dithizone method after acid extraction (see ref. 27).

[b] A preliminary measurement on a ceruloplasmin sample contaminated with a diamagnetic protein gave a value of 740(± 110).

In Table 7 are collected some of the results of magnetic susceptibility measurements on ceruloplasmin and laccase. The volume susceptibility increment was obtained from the difference between measurements on protein solution and on its dialysis buffer. Reduction of samples was achieved by addition, under argon, of 10 μl of 10% $Na_2S_2O_4$ in 1 *N* NaOH to 0.5 ml of protein solution, and this proved effective, since the samples remained colourless until the sample tube of the susceptometer balance was re-opened. In the latter experiments, the same concentration of dithionite as used for reduction was included in the buffer to correct for its diamagnetism and for the changed paramagnetism due to removal of dissolved oxygen. The two columns to the right in Table 7 show calculated differences in molar susceptibility between the oxidized and the reduced forms of the protetins, as well as the differences between blue and leuco ceruloplasmin, respectively, and apoceruloplasmin.

Calculations (see ref. 28) of the molar susceptibility for Cu^{2+} with the orbital paramagnetic contribution found earlier in ceruloplasmin and laccase (81) gave about 1400×10^{-6} c.g.s.e.m.u. The most accurate values of $\chi_M \times 10^6$ in Table 7 are 550 and 570, or about 40% of the calculated value for both proteins.

Discussion

According to the ratios of divalent copper to total copper derived from ESR and chemical data in Table 6, both ceruloplasmin and laccase in the oxidized form have approximately 50% of the total copper in the monovalent state. The deviations from the exact ratio of 0.5 are very probably due to the inexactitude of the method. Thus, the perchloric acid-treatment apparently was not wholly effective in oxidizing the cuprous ions and in liberating the metal, since the samples did not give entirely reproducible values, and the type of signal obtained differed in separate experiments. Corrections were not made in Table 6 for the residual signal obtained from native protein in the presence of a large excess of substrate. It can not be excluded that this signal represented a persisting steady state, at least in experiments with ceruloplasmin (cf. Chapter 6). A more plausible explanation is, however, that the signal originated from denatured protein, and that the denaturation did not significantly change the total absorption. In neither case does the residual signal affect the ratios given in Table 6.

The area under the ESR absorption curve is proportional to the concentration of unpaired spin in a sample, and it is thus obvious that line broadening conditions will introduce a corresponding uncertainty in the estimates (cf. Fig. 30d, where the areas under the four curves are equal). Although this source of error generally is of minor importance in the case of cupric complexes, it is desirable to check the ESR results with the independent method offered by magnetic susceptibility measurements.

The results obtained with this method for ceruloplasmin and laccase (Table 7) exclude the possibility that the ESR measurements were unable to account for bivalent copper because of excessive line broadening. The figures obtained with these methods are reasonably concordant as regards the actual proportion of cuprous and cupric ions.

The conclusion that exactly half the number of copper ions (4 in ceruloplasmin, 2 in laccase) have different valency in the oxidized form of the protein gains support from the experiments by Laurell (8) referred to in the *Introduction*. This inference is strengthened even more by observations on ceruloplasmin indicating that about half the number of copper ions are differently bound to the protein, not only in the oxidized form, but also in the *leuco* form where all copper is monovalent. Thus, Scheinberg and Morell (98) found that about half of the 8 atoms in the molecule were exchangeable with Cu^{64}, provided that sufficient ascorbic acid was present to reduce the protein to the leuco form. Other indications that two different forms of copper exist in ceruloplasmin may be found in refs. 85 and 99.

Assuming equal number of Cu^{1+} and Cu^{2+} in the oxidized form of respectively ceruloplasmin and laccase one next asks how these two forms of copper are related to the properties of the catalysts, particularly their colour, ESR hyperfine structure and activity. It is quite natural to think of these ions in terms of cuprous-cupric pairs. Several instances of low molecular weight compounds are known which contain two (or more) ions of the same metal at two different oxidation stages. These compounds are often deeply coloured, for instance Cu_2Cl_3 (100) which has an absorption band that covers all wave-lengths shorter than 550 $m\mu$. This compound is formed in hydrochloric acid solutions containing copper in the two states of oxidation. If ceruloplasmin (about 5%) is denatured by adding a small amount of HCl to a final concentration of at least 6 *N* and the white precipitate is warmed to about 100°C for a few minutes, the supernatant turns yellow, brown and finally black. The coloured species is, in all probability, a mixed cuprous-cupric chloro-complex, such as Cl—Cu—Cl—Cu—Cl (100). It is seen that this complex is symmetric and contains a bridge ligand. Considering the experiments mentioned in the preceding paragraph, it seems improbable that the cuprous-cupric pairs of ceruloplasmin are symmetric. It has recently been suggested by Blumberg *et al.* (72) that, in ceruloplasmin, the function of the permanently monovalent copper ion in the pair is to act as a donor in a charge transfer mechanism with the cupric ion as acceptor. The two metal ions are, according to this hypothesis, connected with a bridge ligand, and the ions are surrounded by different ligand fields affecting the unpaired electron differently, *i.e.* they form an asymmetric mixed complex (cf. also foot note on p. 2341 of ref. 85). The latter consideration is important, because cuprous-cupric pairs in which the unpaired electron is equally shared between the two copper nuclei would give rise to an ESR hyperfine structure with seven components, which is not the case with the ceruloplasmin spectrum.

The principal reason for assuming that the permanently monovalent copper has a charge-donating function is, according to Blumberg *et al.*, that the optical spectrum of ceruloplasmin and apoceruloplasmin does not show any absorption bands corresponding to excitations in organic groups (ligands) of sufficiently low energy for this function.

Charge-donating organic ligands should, however, be present in azurin, which has a molar extinction coefficient at 625 mμ of 3500 (94). The molecule contains only one copper atom per molecule, which is bivalent under aerobic conditions (29), thus excluding the alternative of a cuprous charge donor. The ESR hyperfine splitting of azurin (29) and of the corresponding protein from *Pseudomonas* (101) is even lower than that of ceruloplasmin and laccase, or 0.006 cm^{-1}, indicating that neither this characteristic necessarily requires a cuprous-cupric interaction (cf. ref. 102).

A possible function of the permanently monovalent copper in connection with the catalytic activity of ceruloplasmin and laccase will be discussed in Chapter 7.

CHAPTER 6

KINETIC EXPERIMENTS

Introduction

The similar substrate specificity displayed by ceruloplasmin and laccase (6) is contrasted by a substantial difference with respect to specific activity. Examples of this are given in the first section of the present chapter.

The valency changes occurring in certain of the copper ions in connection with transformations between blue and leuco forms of ceruloplasmin and laccase suggest that the flow of electrons from donor substrate to acceptor substrate is conveyed by these ions. Results of kinetic ESR experiments are in agreement with this assumption. These results are abstracted from ref. (29) to which the reader is referred for additional details and theoretical considerations. Supplementary data given in the second section were obtained in collaboration with Drs. B. G. Malmström, R. Aasa and T. Vänngård.

A comparison of the catalytic rates of ceruloplasmin and laccase

Results with three substrates having oxidation products suitable for direct spectrophotometric measurements are collected in Table 8 together with pertinent experimental data.

In a second series of experiments the method given by Walaas and Walaas (22) was employed, with the exception that the buffer was 0.1 *M*

Table 8. *Relative specific activity of laccase and ceruloplasmin with three different substrates.*

0.01 *M* solutions of substrates in 0.1 *M* triethylamine-acetic acid buffer (TEA-HAc), pH 5.5, with 4×10^{-5} *M* EDTA, were mixed in a stopped-flow apparatus with an equal volume of respectively 2.2×10^{-3} m*M* ceruloplasmin (Kabi DwZ 134) and 4.2×10^{-5} m*M* fungal laccase A in 0.1 *M* TEA-HAc, pH 5.5. The reults are given as the ratio L/C for each substrate, where L and C are $\Delta A\gamma\times min^{-1}\times[Cu]^{-1}$ with laccase and ceruloplasmin, respectively. L and C were constant during the period of measurement.

Substrate	λ mμ	L/C
p-phenylene diamine	480	830
N,N-dimethyl-*p*-phenylene diamine	552	530
N,N,N',N'-tetramethyl-*p*-phenylene diamine	560	630

triethylamine acetic acid, pH 5.5. Experimental data and results are given in Table 9.

Instrumentation as for activity determinations in *Methods,* Chapter 4.

Comment.

The results presented in Tables 8 and 9 demonstrate a substantial superiority of laccase as a catalyst for all of the substrates tested. The values for di- and tetramethyl *p*-phenylene diamine are probably reasonably accurate, because the measurements were made on a relatively stable first oxidation product. The coloured species determined in the case of *p*-phenylene diamine (PPD) does not represent the first oxidation product (cf. ref. 44). The method used in the experiments summarized in Table 9 tended to give too low values with high rates of product formation, evidently because of a relatively slow reaction rate between product and NADH. This was apparent in the case of *p*-phenylene diamine, where an increasing absorbancy caused by the coloured product was observed. Corrections for the absorbancy of this product at 340 mμ were made in these cases.

Kinetics of the valence-changing copper in ceruloplasmin

The reactions between the oxidized form of the catalyst, ECu^{2+}, and a donor substrate, AH_2 may be written schematically as follows:

$$ECu^{2+} + AH_2 \rightleftarrows ECu^{2+}AH_2 \quad (1)$$

$$ECu^{2+}AH_2 \rightarrow ECu^{1+}(AH_2)^+ \quad (2)$$

$$ECu^{1+}(AH_2)^+ \rightleftarrows ECu^{1+} + (AH_2)^+ \quad (3)$$

and the reaction between the reduced form and the acceptor substrate

$$ECu^{1+} + \tfrac{1}{4}O_2 + H^+ \rightarrow ECu^{2+} + \tfrac{1}{2}H_2O \quad (4)$$

Table 9. *Molecular activity for laccase and ceruloplasmin with four different substrates*

1.5×10^{-4} *M* solutions of substrates in 0.1 *M* triethylamine acetic acid buffer, pH 5.5, containing 2×10^{-4} *M* NADH and 4×10^{-5} *M* EDTA. Catalyst added to the substrate mixture by means of a plastic plunger; final concentrations: laccase 4.7×10^{-6} m*M*; ceruloplasmin 8×10^{-4} m*M*. Temperature 25°C. Cf. ref. 22.

Substrate	Molecular activity (moles of substrate per mole per minute)	
	Laccase	Ceruloplasmin
p-phenylene diamine	12,000	10
catechol	1,700	0.8
pyrogallol	550	1
hydroquinone	210	0.4

Table 10. *Kinetic parameters for ceruloplasmin in 0.1 M triethylamine—acetic acid buffer (pH 5.5) at 17°C, determined by ESR measurements* (29).

Initial concentrations: total copper (c_{tot}), $8 \cdot 10^{-4}$ M; Cu^{2+} (c_o), $4 \cdot 10^{-4}$ M; O_2 (u_o), $2.9 \cdot 10^{-4}$ M; and substrates, $5 \cdot 10^{-3}$ M.

Substrate	$T\frac{1}{2}$ (min)	c_s/c_o	k_1 (min^{-1})	q'_{O_2} (min^{-1})	T_s (min)	q''_{O_2} (min^{-1})	k_2 (min^{-1})
PPD	0.015	0.20	37	0.92	0.4	0.9	9.2
Pyrogallol	0.065	0.47	5.6	0.34	1.4	0.27	5.0
Catechol	0.08	~ 0.48	4.5	~ 0.27	3	0.12	4.2
Quinol	0.4	0.80	0.35	0.035	10	0.036	1.4
Hydroxylamine	—	> 0.7	—	—	> 20	—	—

The formation of an enzyme-substrate complex is suggested by experiments of the type shown in Fig. 11 p. 23. With *p*-phenylene diamine as substrate the product formed (Eqn. 3) was identified by the ESR spectrum as the positive ion. In experiments described in detail in ref. 29 ESR was employed for measurements of the reduction kinetics of ECu^{2+} by various substrates (Table 10). Fig. 34 shows two representative curves of the time course of this reduction obtained with pyrogallol and catechol at 17°C. Three well defined phases are discernible, a rapid initial phase, a steady state phase, and a final decay phase. The time required for half-reduction to the steady state level ($T\frac{1}{2}$), the duration of the steady state (T_s) and the bivalent fraction of the valence-changing copper during the steady state (c_s/c_o) are given in Table 10 for the various substrates. Oxygen was consumed at a practically constant rate until exhausted, judging from the well defined endpoint of the steady state. Accordingly, this rate could be calculated from the known starting concentration and T_s. These values, q''_{o_2} (moles per mole of total copper per minute), are included in Table 10. On the other hand, assuming that both reduction and re-oxidation are first order reactions with the substrate concentrations used (proportionality constants k_1 and k_2, respectively, see Table 10), the oxygen consumption rate, q'_{o_2} can be calculated independently from $T\frac{1}{2}$, c_s and c_o (see ref. 29). The good agreement between the q'_{o_2} and q''_{o_2} values (Table 9) favours the above assumption, thus providing strong evidence for a mechanism involving reduction of Cu^{2+} by the substrate followed by re-oxidation by O_2.

An arrangement was devised for measurements of the rate of re-oxydation of ceruloplasmin and laccase copper after titration with substrate under argon. Values of k_2 obtained spectrophotometrically and by ESR measurements after reduction with ascorbic acid did not agree with the results in Table 10. Thus, if Eqn. 4 were the limiting step in the re-oxidation, the value of k_2 would exceed 150—1500 min^{-1}. On the other hand, if Eqn. 3 were rate limiting, the concentration of the enzyme-product com-

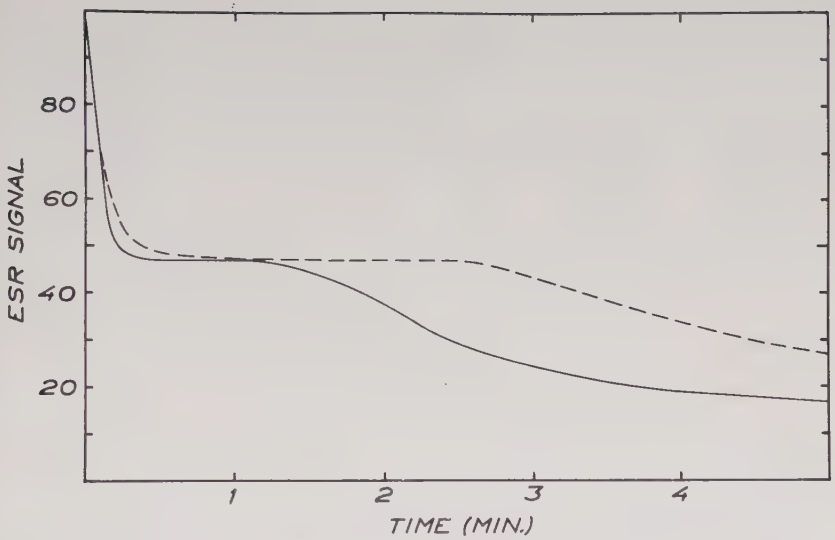

Fig. 34. Decay curves of ESR signal of Cu^{2+} in ceruloplasmin obtained from recordings on high- and low-response recorders. Concentrations immediately after mixing of total copper about $8 \times 10^{-4} M$ and of substrates (solid curve, pyrogallol; dashed curve, catechol) $5 \times 10^{-3} M$. Buffer 0.1 M triethylamine—acetic acid, pH 5.5 (29).

plex would be high. The latter alternative is, however, not very likely (29). The method has not yet been used with any of the substrates included in Table 10. The possibility of an inhibition of the re-oxidation with these substrates must be considered, for example complex formation between the reduced valence-changing copper and the aromatic ring (see Chapter 7).

Numerical integrations of kinetic equations were compared with the experimental curves for Cu^{2+} and for the *p*-phenylene diamine radical. The results indicate that the radical decay occurs by a reaction not involving the enzymes. Numerical calculations of the rate constants for the PPD-radical decay by dismutation gave an about 10 times higher value if 0.15 M NaCl was present (different buffer concentrations were used in the experiments shown in Figs. 4 and 5 of ref. 29, but the same result was obtained in a later experiment with all conditions equal, save for the chloride). The radical concentration calculated numerically in Fig. 5 of ref. 29 has been determined experimentally under the conditions given in the legend (room temperature), and a value of about $1.5 \times 10^{-4} M$ was obtained.

A preliminary experiment has been undertaken with presumptive competitive inhibitors which were chosen according to the hypothesis (ref. 29, cf. Chapter 7) that the role of the permanently monovalent copper of

ceruloplasmin and laccase is to partake in substrate binding by interacting with the π-electron system of the substrate. The experiments were conducted according to the legend of Fig. 34, with a final inhibitor concentration of 5×10^{-3} *M*. It was found that salicylate caused T_s to increase significantly, whereas maleate (cf. ref. 25) and AMP were non-effective.

CHAPTER 7

THE FUNCTION OF CERULOPLASMIN

This chapter is devoted to a few hypothetical considerations based on the results described in the preceding chapters and in the literature.

The role of the unchanging cuprous ions

The discussion in this section refers exclusively to the resting oxidized form of the catalyst and to its interaction with an electron-donating substrate. According to the conclusion arrived at in Chapter 5, the cuprous-cupric interactions assumed by Beinert *et al.* (102) and by Blumberg *et al.* (72) are not necessary prerequisites for establishing respectively a narrow ESR hyperfine structure and a charge-transfer mechanism similar to those in ceruloplasmin and laccase.

It was suggested previously (29) that Cu^{1+} may partake in substrate binding by interacting with the π-electron system of the substrates. It was pointed out that at least two types of facts are in favour of such a hypothesis. Firstly, complexes involving a π-electron system of the ligand are formed preferentially with transition metals in their low valencies (87). Secondly, it has recently been shown (24) that the best substrates of ceruloplasmin are aromatic compounds with a high density of π-electrons in the ring, and there appears to be a direct relationship between the density of π-electrons in the substrate and the rate of the reaction. Furthermore, it was suggested (29) that substrate binding in this manner may result in a delocalization of the donor orbital, thus facilitating the electron transfer. The fact that addition of two (24) and possibly four methyl groups to the amino groups of *p*-phenylene diamine does not alter the reaction rate very much may also indicate that these substrates are both attached to the catalyst and tapped of an electron *via* π-bonding of the aromatic ring. In addition, the general lack of structural consistency among the substrates is compatible with such a "one-point attachment."

The electron-accepting cupric ion (ref. 29 and Chapter 6) has probably a highly delocalized unpaired hole (81) which should allow overlapping with electron-donating orbitals. On the other hand, considerations in Chapter 5 deemed it unlikely that the delocalization reaches as far as to include the cuprous ion. An attractive compromise is suggested by the bridge ligand introduced by Blumberg *et al.* (ref. 72 and Chapter 5).

These conceptions are visualized in Fig. 35.

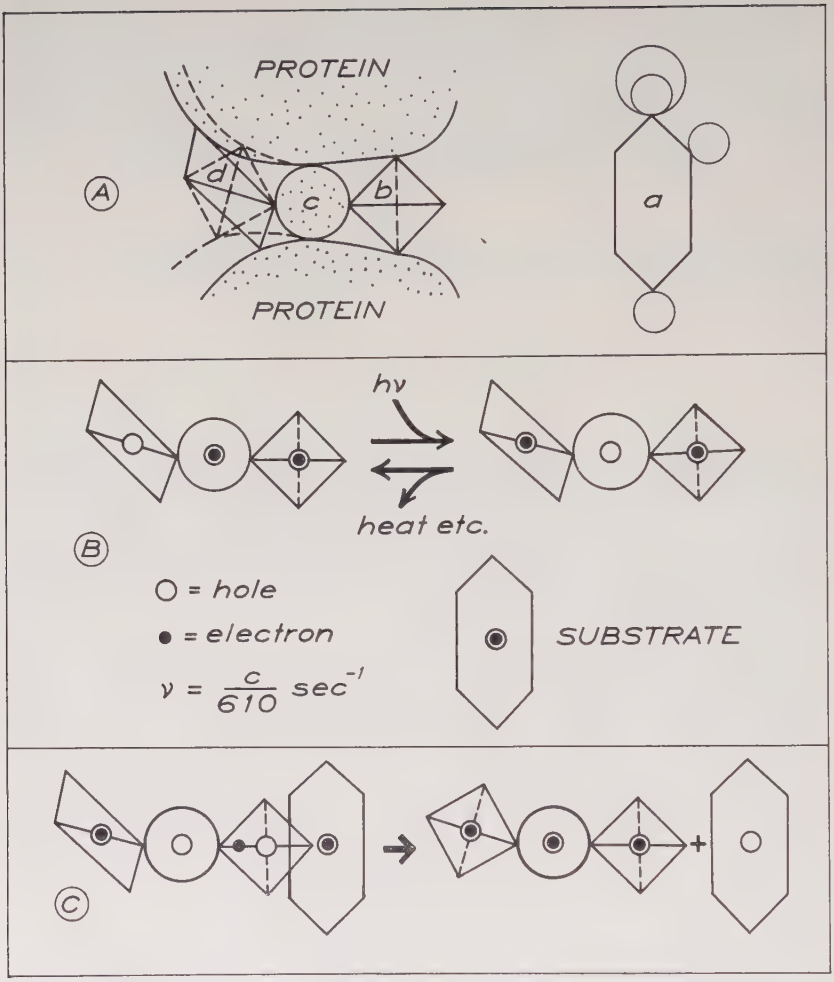

Fig. 35. Model of cuprous-cupric pair in ceruloplasmin and its interaction with a substrate molecule.

A. a, aromatic substrate; b, permanently cuprous ion; c, bridge ligand; d, valence-changing ion (solid line, cupric; dashed line cuprous). The superimposed tetrahedron and skew elongated octahedron symbolize coarsely the abnormal steric conditions; rearrangement of ligands in connection with oxidation-reduction is indicated.

B. Principal parts with relevant holes and electrons indicated. Charge-transfer mechanism in ceruloplasmin absorbing light at 610 mμ. c = velocity of light in mμ per second. h = Planck's constant (energy divided by frequency).

C. Displacement of charges in the enzyme-substrate complex (left) and ejection of free radical (right).

The reduction of oxygen

The following discussion refers to the reduced catalyst and its interaction with oxygen. While the oxidizing phase of the action cycle of ceruloplasmin comprises four separate one-electron transfers, the reducing phase is concerned with a four-electron transfer that may not be separable into independent parallel events. If it proceeded in independent steps, a number of highly active free radicals (and H_2O_2) should appear. However, it is not known whether they do. It is appropriate here to quote H. S. Mason (104): "During the formal stepwise addition of four electrons to molecular oxygen, at least two very reactive radical intermediates, perhydroxyl and hydroxyl, are produced . . . From a biological point of view, reduction mechanisms forming these free radicals are disadvantageous because an energetic random attack upon functionating components of the cell must ensue, and becaunse biochemically efficient utilization of free energy of reaction of these *free* radicals is impossible."

Lack of evidence on this point prompts us to look for indications elsewhere. Certain structural peculiarities of the reactants are at least suggestive. On the one hand, a certain energy expenditure is needed to split the O—O bond. On the other hand, one must consider the fact that the ligand frame of the valence-changing copper is able to accommodate alternately a cupric and a cuprous ion, in spite of their considerably different steric preferences. This could mean that rearrangement energy is exchanged with the tertiary structure of the protein. All four valence-changing ions in ceruloplasmin may be located sufficiently close to one another to react concertedly with one oxygen molecule, activating it by transferring the negative free energy stored in the distorted tertiary structure, and at the same time reducing it completely by each giving off one electron. Similar mechanisms have been discussed for haem-proteins (105, 106). Indications that conformal changes do indeed occur in connection with the functioning of ceruloplasmin have recently been observed by Kasper and Deutsch (14) and by Blumberg *et al.* (72). The fact that laccase has only two cuprous-cupric pairs should be a valuable starting-point in comparative investigations on the acceptor sites of these proteins.

Thus, according to the model suggested here, all of the 8 copper ions in ceruloplasmin are confined to a very small part of the molecule. The axes of the four cuprous-cupric pairs (Fig. 35) converge towards a common acceptor site. The latter may be retracted from the protein surface, since determinations of proton relaxation rates (72) indicate that valence-changing copper is relatively unaccessible to water. Also the donator sites may be more or less buried, judging from the low activity of ceruloplasmin. This assumption gains some support from the observation that there will be a moderate increase of the activity if the protein is exposed to low concentrations of urea (about 0.5 *M*).

A transport hypothesis

It is generally taken for granted that copper to be used in the synthesis of cytochrome oxidase (and dopa-oxidase) is transported in plasma bound to albumin. Albumin is the only plasma protein in the normal individual, besides ceruloplasmin, which is known to be associated with copper. However, considering the fact that this element is as indispensable as iron, it appears more likely that its transport within the organism is controlled through a specific carrier. As a matter of fact, Scheinberg and his collaborators suggested, some years ago, (71) that ceruloplasmin is engaged in the transport of copper by a mechanism similar to the shuttling action of transferrin. The assumption was based on the observation mentioned in Chapter 5 that, *in vitro,* the reduced protein will exchange 4 of its copper ions. This hypothesis was, however, abandoned later on, since the authors were able to prove that no exchange of ceruloplasmin copper occurs *in vivo* (107). But this demonstration does, of course, not exclude that copper is transferred by ceruloplasmin according to some different principle.

Here I shall discuss briefly a transport mechanism that does not seem to have been considered before. As will be shown in the last section, certain less well understood features of Wilson's disease may be explained, if one assumes that *copper can be utilized for the synthesis of cytochrome oxidase only if it is presented to the cell as ceruloplasmin.*

The last few years have witnessed a growing interest in the role of copper in cytochrome oxidase. A recent survey of this controversial problem may be found in ref. 108. The oxidase appears to contain one firmly bound copper atom per haem *a* iron atom, although several authors have claimed a higher copper content. Opinions differ as to the participation of this copper in the electron transport. According to Beinert *et al.* (102), the copper is reduced by cytochrome *c* and reoxidized by oxygen. Atherton *et al.* (103) have recently shown that the oxidation rate is too rapid to be measurable with a technique allowing resolution in the millisecond range. The contradictory results obtained may be due to the fact that cytochrome oxidase is a stroma-bound enzyme which is difficult or impossible to isolate without causing damage. This may well be the case, even if certain functions are preserved. (Cf. *Introduction* of Chapter 2).

According to the present hypothesis the function of ceruloplasmin may be to act as a carrier of copper from the liver to various sites where cytochrome oxidase is synthesized. More precisely, an elaborate prosthetic group comprising 4 cuprous-cupric pairs framed in a system of ligands may be transferred from the large carrier part of the ceruloplasmin molecule (which is catabolized) to the cytochrome oxidase unit under construction. In its final position as the ultimate link of the electron transport system of the mitochondrion the prosthetic group achieves rapid, safe and economic activation and reduction of oxygen. (Cf., however, ref. 103).

Wilson's disease

Clinically, Wilson's disease is characterized by a pronounced variability of symptoms (17). Several of these symptoms can not be explained simply as effects of copper poisoning. The ceruloplasmin concentration in plasma is low, but complete absence of the protein in venous blood is not very common. According to Sternlieb and Scheinberg (109) a concentration lower than 1 mg/100 ml was seen in 27 out of 111 cases. On the other hand, a few cases are known who presented nearly normal values (cf. patient V.C. in Fig. 25). In Sternlieb's and Scheinberg's material 4 cases had concentrations higher than 20 mg/100 ml. The very limited investigation on such a patient described in Chapter 2 did not disclose any substantial qualitative abnormality of the protein. It seems, in fact, improbable that in some patients the disease is caused by a qualitative deficiency and in others by a quantitative deficiency, since only one frequency peak was observed in the material mentioned above. One can therefore assume that *some* production of a normal ceruloplasmin occurs in most, if not all, cases. The concentration of albumin-bound copper is supernormal in most, if not all, cases. This constellation may be explained if it is assumed that there is some kind of block that interferes with the transfer of copper to ceruloplasmin in connection with its synthesis in the liver (cf. ref. 1). The blockage is, however, not complete, and it seems plausible that it is partly removed as a consequence of the increased concentration of albumin-copper. The increased absorption of alimentary copper is apparently caused by some regulatory mechanism which is activated by the subnormal ceruloplasmin concentration (or by some consequence of the latter), while being insensitive to the immediate result of the regulatory stimulation, *viz.* the increased albumin-copper concentration. Thus, ceruloplasmin must have indispensable properties which can not be substituted for by albumin-copper. But the properties of ceruloplasmin, regarded simply as a plasma constituent, do not strike one as particularly important; just an ineffective oxidase in the wrong place, lacking a suitable substrate. The patho-physiological situation does, however, lend itself to ready explanation if it is assumed that ceruloplasmin represents a unique transportation arrangement which is necessary for the production of an intracellular copper enzyme that is of unquestionable importance. The only known important copper enzyme in man is cytochrome oxidase.

From this inference we deduce: 1) some symptoms in Wilson's disease should be due, paradoxically, to copper deficiency, 2) such symptoms should be less pronounced in cases with a relatively high ceruloplasmin concentration in plasma.

Man's food, though sometimes bizarre and often miserable, is evidently never sufficiently extreme to produce uncomplicated copper deficiency. This condition is well known in animal pathology, because there exist extensive copper-deficient areas which are used as grazing grounds in sheep-farming and cattle-breeding. Failures in these industries as well as syste-

matic experimental work with a range of animal species have shown that copper deficiency produces a wide variety of symptoms (see, for example, ref. 110). Thus, lambs born of copper-deficient ewes develop lesions of the central nervous system, such as symmetrical cerebral demyelination, degeneration of motor tracts in the spinal cord and changes of the neurons of the brain stem. These various lesions occur concomittantly with a decreased content of cytochrome oxidase.

Another lesion in copper deficiency with a counterpart in Wilson's disease occurs in the bone tissues. The bones of calves and lambs living in areas with copper-deficient pastures tend to fracture easily. Similar effects can be produced experimentally in various species, for example in dogs (111) who develop a diffuse osteoporosis with no primary disturbance of the calcification mechanism. In Wilson's disease Finby and Bearn (112) showed roentgenographically that 14 out of 20 patients had a variety of osseous lesions. The authors considered it unlikely that all of the bone lesions seen in Wilson's disease could be related to the concurrently disturbed renal function (see below) and concluded that "the possibility that the skeletal abnormality may be directly due to abnormal copper metabolism must be considered . . . It is not immediately apparent how these observations in (copper-deficient) animals are related to the present findings in patients with Wilson's disease in whom an increased total body copper is ordinarily found."

Unfortunately, studies of the kidney function in copper deficiency do not appear to have been carried out. In Wilson's disease the copper content of the kidneys is increased, but anatomic defects are usually not detectable. Clinically, a variety of functional derangements are more or less constantly observed, comprising amino-aciduria, uricosuria, glycosuria, albuminuria and hypercalciuria. These functional impairments of the kidneys in Wilson's disease are generally assumed to be caused by toxic effects of deposited copper. Considered on the basis of the suggested transport hypothesis some of these functional drangements may, however, be explained as due to a failure of the mitochondrial production of ATP which is required in active transport processes. In a material presented by Bearn and Kunkel (113) no glycosuria was observed in patients with a ceruloplasmin concentration higher than 24% of the normal value (7 patients), whereas 5 out of 7 patients with lower ceruloplasmin concentrations showed varying degrees of glycosuria (percentage calculated from last column of Table I and normal mean value given in first column of Table II, ref. 113). This finding is in accordance with deduction 2.

Also in accordance with the second deduction is the circumstance that patients with a high ceruloplasmin concentration have only mild motor disturbances or may even show normal nervous function (see a survey in ref. 18). It should be noted that this variant of Wilson's disease is comparatively rare (114), which makes the positive correlation with the equally rare high-ceruloplasmin variant all the more impressive.

It was mentioned earlier that cytochrome oxidase can display activity

in the laboratory without conclusive signs of participation of the copper moiety. This circumstance does not, of course, allow any conclusions about *in vivo* conditions. It seems rather probable that cytochrome oxidase is not synthesized in the absence of copper. Lemberg *et al.* (115) postulate that a copper enzyme is involved in the synthesis of cytochrome oxidase and suggest that "it is even possible that the copper enzyme required for the haem *a* synthesis becomes incorporated in the cytochrome oxidase."

Summary

1. Ceruloplasmin in human serum has been analyzed by gradient chromatography on hydroxylapatite at neutral pH in combination with a spectrophotometric assay method that depends on the catalytic properties of the protein.

2. The chromatographic analysis showed that human ceruloplasmin is heterogeneous. Two distinct active components were always found in fresh sera from normal adults. The relative abundancy of these components varied between 6 : 1 and 3 : 1. Nothing has emerged that indicates a non-biological cause of this heterogeneity.

3. Sera from normal newly born individuals and from patients with Wilson's disease (hepatolenticular degeneration) also contained two active ceruloplasmin components. The chromatographic behaviour and the relative abundancy did not differ significantly from the normal. This fact, together with other considerations, suggest that ceruloplasmin is only quantitatively abnormal in Wilson's disease.

4. Additional chromatographic heterogeneity of catalytically active ceruloplasmin can be brought about by various treatments (storing, freezing and thawing, chromatography on DEAE ion exchangers). The three main components appearing in hydroxyl apatite chromatograms under these conditions were different as regards stability, but did not differ significantly with respect to sedimentation constant, copper content, copper valency or specific activity.

5. A semicontinuous method has been devised for purification of ceruloplasmin directly from human serum. The method comprises two protein-protein displacement chromatographies and one gel filtration step, and insufficiently purified fractions are reutilized. The recovery is about 85%, and about 80 mg of a practically pure ceruloplasmin is produced daily with a moderately dimensioned apparatus.

6. Studies by means of ESR and magnetic susceptibility of human ceruloplasmin and fungal laccase show that only about half the number of copper ions are cupric in the oxidized form of both catalysts, whereas practically all copper is monovalent in the reduced form of these metalloproteins.

7. ESR experiments on ceruloplasmin involving controlled urea denaturation indicate that the catalytic activity and the characteristic blue colour of the protein are intimately connected with the specific bonding of the valence-changing copper ions.

8. Kinetic ESR experiments gave further evidence for the direct participation of the valence-changing copper ions in oxidation-reduction processes which are catalyzed by ceruloplasmin.

9. The specific activity of fungal laccase is 500 to 1000 times higher than that of human ceruloplasmin with 6 common substrates, according to spectrophotometric determinations.

10. The structure and function of the active sites are discussed. It is assumed that one valence-changing and one permanently monovalent copper ion are separated by a common ligand, constituting a cuprous-cupric pair, and that four such cuprous-cupric pairs are confined to a small part of the molecule with their valence-changing ions sufficiently close to form a common acceptor site which activates and reduces the oxygen molecule in a concerted reaction. The permanently cuprous ions may form four mutually independent sites, each capable of binding one donor substrate molecule. The electron transfer involved in such a bond is thought to be the first step in the oxidation phase of the catalytic cycle.

11. A new hypothesis for the physiological function of ceruloplasmin is presented. It is suggested that this protein represents a transport form of a complicated copper-containing prosthetic group which acts as an oxygen-activating unit in cytochrome oxidase. This assumption would appear to explain certain less well understood features of Wilson's disease.

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Price Sw. kr 20: —

Cover printed by Almqvist & Wiksells Boktryckeri AB. Uppsala 1964